1999

Development of Efficient Rice DNA Transformation Methods and Rapid Evaluation of Transgenic Lines.

Junda Jiang
Louisiana State University and Agricultural & Mechanical College

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

DEVELOPMENT OF EFFICIENT RICE DNA TRANSFORMATION METHODS AND RAPID FIELD EVALUATION OF TRANSGENIC LINES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Agronomy

by
Junda Jiang
B. S., Zhejiang Agricultural University, China, 1987
M. S., Zhejiang Agricultural University, China, 1990
December 1999

Bell & Howell Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

DEDICATION

To my father Minghui Jiang and my mother Linwen He, a typical couple of rice farmers in China.

ACKNOWLEDGMENTS

It is an amazing grace that I have been allowed since the Fall 1995 to come to America to study, work, live, and foremostly learn to believe in God, here far away from my home country China. I dreamed of studying abroad for a Ph.D. degree as early as 15 years ago when I was still a sophomore in China, but I had never thought of it coming true until I entered LSU, with the recommendation of Dr. Qiren Chu, my former supervisor in China and currently also serving as one of my committee members. Never to mention that I had ever thought of believing in God, blinded in the past. I can not help first of all praising: "Thank you, Lord, for everything you have done for me and my family, for giving your guidance and protection during the toughest time of my life, and for giving me strength, perseverance, and enthusiasm in accomplishing this big task and as well as challenges in daily life. You are the origin of life, love, and help I am experiencing".

I sincerely thank my two co-major professors Dr. James H. Oard and Dr. Steve D. Linscombe for their great support, advice, encouragement, and understanding throughout this study at the Louisiana State University A&M College and the LSU Agricultural Center. Without their thoughtful arrangements, instructions, and help in the laboratory and in the field, it would be impossible for me to complete any portion of this study. I gratefully appreciate my dissertation committee members, Dr. James L. Griffin, Dr. Stephen A. Harrison, Dr. Donald E. Groth, and Dr. Qiren Chu for their constructive advice, critical readings, and support that made my work significantly better. My thanks

are also extended to Dr. Joseph Woodring for his acting as the Dean's representative in assistance and supervision of progress of my graduate program.

I am especially indebted to Dr. Qiren Chu for his advice, encouragement, and considerable assistance in my academic career including his recommendation and help that made possible my enrollment at Louisiana State University, his unreservedly teaching me of rice anther culture technique with his superior expertise in the rice biotechnology lab of the LSU Rice Research Station at Crowley, and his generously providing me with several rice cell lines used as a portion of experimental materials in this study.

I am greatly thankful to Dr. Joseph A. Musick, the Resident Director of the LSU Rice Research Station at Crowley, for his offering me a Graduate Research Assistantship from August 1995 to February 1998, and Dr. Freddie A. Martin, the Head of the LSU Agronomy Department, for his offering me a position as Research Associate Specialist since March 1998. These opportune help enabled me to pursue the Ph.D. degree and continue to join the LSU Agricultural Center during this period of time.

The author wants to express his sincere gratitude to Agronomy faulty Dr. Bradley C. Venuto, Dr. Scott B. Milligan, Dr. Manjit S. Kang, Dr. Gerald O. Myers, and Dr. Paul Bell for giving many helpful suggestions on his work during the preparation and progress of this dissertation. The author also appreciates Dr. Norimoto Murai of the LSU Plant Pathology and Crop Physiology Department for his gift of plasmid pTRA151 and his suggestions on this study, Dr. Eric Achberger of the LSU Biological Sciences for his helpful discussion on construction of the plasmid pPAT63.

The author is thankful to the supporting staff of this department, especially Ms. Connie Rozarto, for their kindest assistance during his graduate study. The author acknowledges that the former Agronomy technician Timothy Pepenburg, undergraduate students Dorsey Johns, Curt Riche, and student worker Delroy Armstrong provided excellent technical assistance in the laboratory, greenhouse, and field experiments and data collection. Ms. Cindy Henk, a Research Associate of Biological Sciences helped him a lot in using dissecting microscopy, taking photographs, and developing films.

The author is grateful to his friends and colleagues, Indira Ratnayaka, Jianlin Wang, Yiming Jiang, Marsha N. Samuels, Chism Craig, Chris Wildman, Alan Shadow, Alicia Ryan, Fabian Capdevielle, Gabriel Aluka, Shaomian Yao, Huangjun Lu, and many more for their cheerful discussions, help, and friendships during his graduate career. My deepest thanks finally go to my wife Ying Chen for her understanding, sacrifice, help, encouragement, and caring love throughout all my endeavors. To her I am forever indebted.

The author acknowledges the contributions of the graduate school editor, Mrs. S. C. Dixon, for her help in the final revision of this manuscript.

This dissertation research was supported by the Louisiana Agricultural Experimental Station and the Louisiana Rice Research Board.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

A rapid and efficient method was developed for production and field evaluation of transgenic herbicide resistant elite U.S. rice (*Oryza sativa* L.) lines and cultivars. Six elite U.S. rice lines, including the cultivar Cocodrie, were transformed for glufosinate (phosphinothricin) herbicide resistance by particle bombardment of mature seed-derived embryogenic calli with a synthetic *pat* gene or the *bar* gene from *Streptomyces hygroscopicus*. By utilizing optimized media for embryogenic callus induction and bialaphos or hygromycin B as a selection agent, 258 independent transformed calli, producing 1268 transgenic plants, were recovered from 5201 bombarded calli. Transformation efficiency ranged from 5% for breeding line LA9502065 to 100% for Cocodrie, with an average across 6 lines of 24 % (1268 plants/5201 calli). Southern blot analysis of genomic DNA isolated from primary R0 and R1 progeny plants demonstrated that the *pat* and *hph* genes were stably integrated into the rice genome. Glufosinate resistance in R0 primary transgenic plants and R1-R4 progeny was confirmed in the greenhouse and under field conditions within two years. Both Mendelian and non-Mendelian patterns of gene segregation for herbicide resistance were observed. Homozygous transgenic lines of Cocodrie and LA9502065 that exhibited desirable agronomic traits were recovered but abnormal phenotypes were also observed in R2 progeny and further confirmed in R3-R4 progeny. The high efficiency and reproducibility of the improved transformation system should make it possible to routinely introduce genes of interest into any elite U.S. rice breeding line.

An alternative efficient rice transformation procedure was established by using *Agrobacterium tumefaciens* strain LBA4404 (pTOK233) and scutellum- or anther-derived calli of two U.S. elite rice lines, LA9502065 and Cocodrie. In a preliminary field trial of transgenic lines, a majority of R2 progeny exhibited normal agronomic traits and less morphological variation than those produced from bombardment experiments, indicating *Agrobacterium* could be utilized for introduction of useful genes into rice. Transgenic rice plants were produced from haploid anther-derived calli or cell suspensions via particle bombardment or *Agrobacterium*. Problems of albinism and sterility of transgenic plants should be addressed in future studies to utilize the potential advantages of anther culture for rice transformation.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world and is the staple food for approximately 3 billion people, predominately in developing countries (Toenniessen, 1996). There are two cultivated species of rice, *O. sativa* and *O. glaberrima*. *O sativa*, the Asian rice, is cultivated worldwide, while *O. glaberrima*, the African rice, is grown on a limited scale in West Africa (Khush, 1997). The varietal types of *O. sativa* are generally classified into *indica*, tropical and temperate *japonica*. Of these, *indica* contributes approximately 80% of the cultivated rice and is often thought of as tropical long-grain rice (Swaminathan, 1982). In contrast, temperate *japonica* rice tends to have short or round grains and is adapted to temperate climates. The tropical *japonica* cultivars, often called "*javanica*", are commercially grown in the Southern U.S. The tropical *japonica* rice tends to have long grains and a morphologically distinct phenotype that was originally classified as part of the *indica* subspecies.

Rice is also important as a model plant for studies of genome and the regulation of monocot gene expression similar to *Arabidopsis* in dicots (Izawa and Shimamoto, 1996). Rice is a simple diploid (2n=24) and has a relatively small genome (4.3 megabases), one-tenth of the size of the human genome and three times that of *Arabidopsis thaliana*. A better understanding of the genetics and the biology of rice can therefore help to improve other cereal crops (Shimamoto, 1995).

Rice improvement through conventional breeding methods has been met with considerable success. Because of consistent efforts by plant breeders, rice production has doubled between 1966 and 1990, but it must increase another 60% by 2025 to feed the

additional rice consumers (Khush, 1997). New developments in two areas of rice molecular biology, namely genome analysis and genetic transformation, have potential to greatly influence future improvement of this important crop (Shimamoto, 1995). This research is intended to supplement the conventional breeding programs aimed at rice improvement by way of providing a breeding line having the desired traits, such as resistance against disease, insects, herbicides, salinity, drought, and improvement of the nutritional quality of rice (Toenniessen, 1995). The first transgenic rice plants were reported in 1988 by three independent groups utilizing protoplasts and electroporation or PEG-inducing methods (Toriyama et al., 1988; Zhang et al., 1988; Zhang and Wu, 1988). Since that time, tremendous progress has been made in this field as reflected by accumulating reports of rice transformation (see Ayers and Park, 1994; Tyagi et al., 1999). Table I.1 illustrates, in chronological order, milestones on achieving and evaluating transgenic rice in the past decade. In terms of methodology development, transgenic rice plants have been produced by protoplast transformation, particle bombardment (Christou et al., 1991), and *Agrobacterium* (Hiei et al., 1994) which was thought impossible several years ago. The first field test of transgenic rice exhibiting an agronomic trait of interest was reported by Oard et al. (1996). In terms of application of rice transformation systems for crop improvement, transgenic rice plants with valued agronomic traits, such as resistance to the glufosinate herbicide (Cao et al., 1992; Christou et al.,1991; Datta et al., 1992; Oard et al., 1996), the sheath blight pathogen, *Rhizoctonia solani* (Datta et al., 1999; Lin et al., 1995), the bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae* race 6 (Song et al.,1995; Tu et al.,1998; Tang et al.,

Table I.1. Historical developments in rice transformation.

Result	Target cells or tissues	Gene transfer method	Reference
Transient activity	Protoplasts	PEG	Ou-Lee et al., 1986
Stable callus-*japonica*	Protoplasts	PEG	Uchimiya et al., 1986
First transgenic *japonica* rice	Protoplasts	Electroporation	Toriyama et al., 1988
	Protoplasts	Electroporation	Zhang et al., 1988
	Protoplasts	PEG	Zhang and Wu, 1988
First transgenic *indica* rice	Protoplasts	PEG	Datta et al., 1990
Variety-independent method	Immature embryos	Particle bombardment	Christou et al., 1991
Agrobacterium transformation	Scutullum-derived calli	Agrobacterium	Hiei et al., 1994
First successful field trial with a useful gene (herbicide resistance)	Immature embryos	Particle bombardment	Oard et al., 1996

1999), rice dwarf virus (Zheng et al., 1997), rice tungro bacilliform or spherical virus (Kloti et al., 1999; Sivamani et al., 1999), rice yellow mottle virus (Pinto et al., 1999), nematode (*Meloidogyne incognita*) (Vain et al., 1998), pink stem borer (*Sesamia inferens*), stripped stem borer (*Chilo suppressalis*), leaf folder (*Cnaphalocrocis medinalis*), yellow stem borer (*Scirpophaga incertulas*) (Cheng et al., 1998; Datta et al., 1998; Duan et al., 1996; Fujimoto et al., 1993; Maqbool et al., 1998; Nayak et al., 1997; Tang et al., 1999; Wu et al., 1997;), tolerance to water deficit and salt stress (Xu et al., 1996), chilling tolerance of photosynthesis (Yokoi et al., 1998), and exhibiting C_4

photosynthesis (Ku et al., 1999) have been reported. However, it should be noted that though transgenic rice plants were firstly produced by protoplast transformation systems, with DNA uptake mediated by electroporation or polyethylene glycol (PEG) treatment (Datta et al., 1990, 1992; Fujimoto et al., 1993; Hayashimoto et al.,1990; Rathore et al., 1993; Shimamoto et al., 1989; Toriyama et al., 1988; Zhang and Wu, 1988), the method suffers from many limitations and is often restricted to certain *japonica* genotypes in which the regeneration system from protoplasts is well established. Rice protoplast isolation is often restricted to embryogenic cell suspensions. It is very difficult to initiate and maintain these cultures and the regeneration capacity of these cultures has been shown to decline gradually with increasing age of the cultures. (Jahne et al., 1995). Furthermore, plant regeneration from protoplasts is labor-intensive, inefficient, time consuming, and is strongly genotype-dependent. The biolistic method (particle bombardment) has emerged as a simple and promising alternative for elite rice transformation (Christou et al., 1991, 1992, 1995). The method is based on high-velocity bombardment of plant cells with DNA-coated microprojectiles (tungsten or gold) accelerated by gun powder discharge or pressurized helium or electronic current. The method is claimed to be genotype independent with the use of immature embryos as target tissues (Christou, 1995, 1997; Datta et al., 1998). However, variable transformation efficiency has been reported by different workers (Christou et al., 1991; Cooley et al., 1995; Li et al., 1993). Preparation of a large quantity of immature embryos is labor-intensive and is environmental- and season-dependent, which makes year-round transformation impossible. Therefore, establishment of an efficient bombardment-

mediated transformation with other types of target tissues is warranted. More recently, fertile *japonica* rice was produced by *Agrobacterium*-mediated transformation of callus tissues from the scutella of mature embryos (Hiei et al., 1994). This pioneering work represents a major breakthrough in rice biotechnology. Monocots, including rice, are thought to be a poor host for *Agrobacterium* infection. The *Agrobacterium* approach has advantages of precise transfer of transgenes of relatively large size and does not require specific equipment. Although several laboratories have reproduced results of Hiei et al. in *indica*, *japonica*, and *javanica* varieties (Aldemita and Hodges, 1996; Cheng et al., 1998; Dong et al., 1996; Rashid et al., 1996), the protocol is not yet routine and needs to be expanded to more genotypes including elite rice cultivars. In addition, no data are available to date of field performance of transgenic rice produced by *Agrobacterium*.

Rice is an important crop in Louisiana, both historically and economically. Disease, insect, and weeds are the primary factors influencing rice production. Recently, developing herbicide resistance in cultivated rice has become an important breeding objective for weed control. One of the major weeds in the southern United States including Louisiana is red rice (*Oryza sativa*). The red rice kernel has a red pericarp and is a noxious contaminant of rice during milling. Red rice competes with white rice by growing tall, and reseeds itself by early shattering of the grains from the panicle. Red rice seeds can be dormant and remain in the soil for many years before germination. It cannot be controlled in commercial rice fields by currently available, broad-spectrum, herbicides (McKenzie et al., 1987). Genetic transformation of rice for herbicide resistance in rice (Cao et al., 1992; Christou et al., 1991, 1992; Datta et al. 1992), and the first field tests of

transgenic rice lines with a herbicide-resistant *bar* gene (Oard et al., 1996) conducted at the Rice Research Station at Crowley, has demonstrated tremendous potential in controlling weed problems.

Toward a long-term goal of applying gene transfer technology to enhance the rice breeding program, substantial efforts on genetic transformation of rice have been made in Louisiana (Hayashimoto et al., 1990; Li et al., 1990, 1992). However, PEG-mediated protoplast transformation system was mostly limited to *japonica* rice cv. Taipei 309 and Nipponbare (Hayashimoto et al., 1990; Li et al., 1990). Even in the case of the transgenic U.S. cultivar Labelle, the foreign gene transferred was a hygromycin-resistant gene (Li et al., 1992). Like the majority of rice cultivars in the U.S., elite Louisiana rice lines mostly belong to tropical *japonica* subspecies. According to our preliminary experiments, the regeneration capacity of Cypress, a typical U.S. elite long-grain cultivar, from explants such as mature embryo and immature embryo-derived calli was very low, compared to the *japonica* cultivar Taipei 309. Immature embryos have been previously used to produce transgenic rice by particle bombardment (Christou et al., 1991; Oard et al., 1996), but their production is labor extensive and dependent on favorable growing conditions in the field or greenhouse. The lack of efficient regeneration methods for tropical *japonica* rice has hindered the general use of transformation technology in Louisiana rice improvement.

The objectives of the present study are: 1) to develop a routine and efficient transformation system for elite U.S. rice lines by particle bombardment of mature seed-derived calli, independent of season and environment; 2) to produce a large number of elite glufosinate-resistant transgenic lines and study segregation of glufosinate resistance

and expression of transgenes under field conditions; 3) to develop transgenic rice plants from elite U.S. rice lines via *Agrobacterium*-mediated transformation and evaluate major agronomic traits of their progeny under field conditions; and 4) to evaluate competence of haploid, anther-derived calli and cell suspensions for both particle bombardment and *Agrobacterium*-mediated transformation and establish a novel, rapid rice transformation system.

CHAPTER 1. REVIEW OF LITERATURE

1.1 Genetic Transformation of Major Agronomic Crops

The first transgenic plants capable of Mendelian transmission of introduced genes were produced more than 15 years ago (Fraley et al., 1983; Herrera-Estrella et al., 1983; Otten et al., 1981). Since then, numerous plants have been engineered to contain genes that enhance disease resistance and herbicide tolerance, delayed ripening, and altered flower color, morphogenesis, or oil quality (Owens, 1995). In 1994 the first genetically engineered major crop, bromoxynil-resistant (BXN) cotton (*Gossypium hirsutum*), was deregulated by the U.S. Department of Agriculture for commercial production. Shortly thereafter, the first genetically engineered food product, the slow-ripening Flavr-Savr® tomato (*Lycopersicon esculentum*), was similarly approved for marketing. Listed in Table 1.1 are examples of transgenic crop cultivars released commercially or under field tests. These historic milestones foretell a future in which gene transfer will be used increasingly for the introduction of important agronomic traits into crops. These advances were made possible with the development of efficient gene delivery technology. This review evaluates the three most commonly used DNA delivery methods: *Agrobacterium*-mediated transformation, protoplast transformation, and particle bombardment, with an emphasis on their role in the creation of transgenic important agronomic crops, particularly rice. Advantages and disadvantages of each method are discussed.

1.1.1 *Agrobacterium*-mediated transformation

The era of plant transformation was initiated by recognition that the soil bacterium *Agrobacterium tumefaciens* may transfer a portion of its DNA (T-DNA) to plants (Barton

Table 1.1. Examples of transgenic crop cultivars released commercially or under field tests.

Trait	Crop	Name	Company	Product status
Quality (vine-riped flavor, shelf life	Tomato	Flavr Savr	Calgene	Released 1994
Quality (vine-riped flavor, shelf life	Tomato	Endless Summer	DNA Plant Technology	Blocked for patent claims
Oil characteristics	Canola	Laurical	Zeneca	Released 1995
Virus resistance	Tobacco Tomato	-	China	Released 1993-1994
Virus resistance	Squash	Freedom II	Asgrow	Released 1995
Insect resistance	Cotton Potato Maize	Bollguard NewLeaf YieldGuard	Monsanto	Released 1996-1997
Insect resistance	Maize	Maximizer	Ciba Seeds	Released 1996
Herbicide resistance	Flax	Trffid	University of Saskatchewan, Canada	Released 1995
Herbicide resistance	Cotton	BXN	Calgene	Released 1994
Herbicide resistance	Canola Corn	Innovator Liberty Link	AgrEvo	Released 1995-1996
Herbicide resistance	Soybean Canola Cotton	Roundup Ready	Monsanto	Released 1995-1996
Herbicide resistance	Soybean Corn	Roundup Ready Liberty Link	Pioneer	Released 1996-1997
Male sterility hybrid system	Canola	-	Plant Genetic Systems	Approved 1996 (USDA, FDA)
Herbicide resistance	Rice	Liberty Link	AgrEvo	Field tested 1993
Herbicide resistance	Rice	Roundup Ready	Monsanto	Field tested, 1998

et al., 1983; Caplan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983). Initial successes were limited to the *Solanaceae*, tobacco (*Nicotiana tobacum*) in particular. Currently a wide range of dicot plants, many of which are of agronomic importance, such as cotton, flax (*Linum usitatissimum*), soybean (*Glycine max*), tomato, and potato (*Solanum tubersum*), have been successfully transformed using *Agrobacterium* (see review by Lindsey, 1992). This technology has allowed the transfer of genes for resistance to viruses (Abel et al., 1986; Cuozzo et al., 1988; Harrison et al., 1987; Lawson et al., 1990; Nelson et al., 1988), herbicides (De Block et al., 1987, 1989; Droge et al.,1992; Fillatti et al., 1987; Hoevan et al.,1994; Shah et al.,1986b; Wohllebaen et al.,1988), insects (Fischhoff et al.,1987; Hidler et al., 1987; Perlak et al.,1990; Vacek et al., 1987), and has also allowed the transfer of genes that improved seed protein quality (Altenbach et al., 1989; De Clercq et al., 1990).

Major advances contributing to the popularity of *Agrobacterium*-based transformation systems include: 1) the development of disarmed strains where the oncogenes, responsible for tumor formation, are deleted and replaced by other genes of interest (Zambryshi et al., 1983); 2) the development of binary vectors, in which the T-DNA borders are located on a small, but wide-host range plasmid and the virulence genes of the Ti-plasmid are located on an independent plasmid and act in *trans* to affect the excision of the T-DNA from the vector plasmid. Virtually any DNA fragment ≤ 150 kb in size can be transferred from the Ti plasmid of *Agrobacterium tumefaciens* (or Ri plasmid from *A. rhizogenes*) as long as it is located between the T-DNA borders Ti/Ri plasmids. These small plasmids can be easily manipulated in *E. coli* and *Agrobacterium.*

As a result, disarmed strains carrying Ti plasmids with foreign DNA cloned in between the T-DNA borders can be used to produce transgenic plants that differ from the wild-type plants only by the presence of foreign DNA instead of tumor formation.

Advantages of *Agrobacterium*-mediated gene transfer over other methods include: 1) high efficiency of transformation; 2) transfer of pieces of DNA with defined ends; 3) transfer of relatively large segments of DNA; 4) absence of a requirement for protoplast culture techniques; and finally, in contrast to direct DNA uptake, transfer of T-DNA and its integration into the recipient genome involves only DNA sequences flanked by border regions and is less subject to intra- and intermolecular rearrangements and usually results in the insertion of a single unrearranged copy of genes of interest (Klee et al., 1987; Songstad et al., 1995). Therefore, this type of gene transfer is normally the method of choice when more than one method is available.

An obvious limitation of *Agrobacterium* was its relatively inability to transform monocots, particularly cereals because of lack of a wound response (Potrykus, 1990). Although there have been a few publications describing *Agrobacterium*-mediated gene delivery to moncotyledonous plants such as *Asparagus officiallis* (Bytebier et al., 1987), *Gladiolus* (Graves & Goldman, 1987), maize (*Zea mays*) (Gould et al., 1991; Grimslay et al., 1988; Schlappi and Hohn, 1992), wheat (*Triticum aestivum*) (Hess et al., 1990; Mooney et al., 1991), and rice (Raineri et al., 1990), use of *Agrobacterium* for routine and reproducible monocot transformation was far from commonplace due to low infection efficiency.

The ability of *Agrobacterium* to transform monocots has been the subject of a serious debate for some time (Potrykus, 1990), since these plants are not natural hosts of this bacterium. Chan et al. (1993) first reported the production of fertile transgenic rice plants from immature embryos infected with *Agrobacterium* in the presence of potato suspension cultures that was rich in the phenolic signal compound acetosyringone and sinapinic acid. However, skepticism still prevailed over the effectiveness of *Agrobacterium* as a suitable method of gene delivery in rice. This controversy was resolved by a convincing report of Hiei et al.(1994) who achieved transformation in *japonica* rice using various explants. A number of strains were constructed, namely, LBA4404 (pTOK233), LBA4404 (pIG121Hm), EHA101 (pIG121Hm), and EHA101 (pTOK233). Of the various explants and strains tested, scutellum-derived calli were the most suitable explants and LBA4404 (pTOK233) was found to be the most effective strain. The authors mentioned several necessary requirements for successful transformation such as the use of acetosyringone and a temperature of 22 °C to 28°C during co-cultivation. Stable integration of foreign DNA in rice chromosomes in a process mediated by *A. tumefaciens* was clearly demonstrated, as well as Mendelian transmission of transgenes to the progeny. Vijayachandra et al. (1995) proved that rice scutella and scutellum-derived calli could induce expression of *vir* genes, but that induction was greatly enhanced by acetosyringone. Following the report of Hiei et al. (1994) and availability of these stains worldwide, several laboratories have reproduced their results in *indica*, *japonica*, as well as *javanica* rice (Aldemita and Hodges, 1996; Cheng et al., 1998; Dong et al., 1996; Rashid et al., 1996;). In addition, Toki (1997) has

developed a new and efficient binary vector (pSMABuba) contained in EHA101 for rice transformation. More significantly, Komari et al. (1996) designed several unique plasmid that carry two separated T-DNA segments, one carrying the nonselectable marker gene (*gus*) and the other carrying the selectable gene (*hph*), in the same plasmid leading to generation of marker-free transgenic plants. More recently, there have been unequivocal reports of stable transformation of maize (Ishida et al., 1996), barley (*Hordeum vulgare*) (Tingay et al., 1997; Wu et al., 1998) and wheat (Cheng et al., 1997) via *Agrobacterium*. These advances are important in at least three ways. First, cell culture and selection steps using *Agrobacterium* are much simpler than protoplast transformation and the particle bombardment approach. Second, the same transformation vector system can now be used both for dicots such as *Arabidopsis* and tobacco and for monocots such as rice, wheat, barley, and maize. Lastly, this method does not require any special equipment, such as a costly biolistic system for particle bombardment. The well-known Japanese molecular biologist Shimamoto stated that "This is particularly important for the progress of rice biotechnology in many rice-growing countries. This transformation method will likely open up a new era for the genetic engineering of rice" (Shimamoto, 1995).

Various factors affect the ability of *Agrobacterium* to transfer foreign genes to the plant genome and must be optimized for successful transformation. They include the nature and physiological state of starting materials, virulence of bacteria, and conditions of co-cultivation and tissue culture. The nature of plant tissues as starting materials predetermines the susceptibility for bacterial infection and regeneration ability of transformed cells. For instance, of different parts of the rice plant, only rice scutella were

found to have ability to induce the *vir* gene of *Agrobacterium* (Vijayachandra et al.,1995). Only calli induced from scutella and immature embryos were highly competent for *Agrobacterium* transformation (Chan et al., 1993; Hiei et al., 1994). Pretreatment of tissues, for example, by preculturing, wounding or enzymatic digestion of cell walls (Mooney et al., 1991), was found efficient in enhancing transformation. The virulence of *Agrobacteria* must be preinduced prior to co-cultivation. To obtain optimal *vir* induction, particular conditions must be met: (1) the pH of the medium must be between 5 and 6; (2) the temperature must be between 20 °C and 30 °C; (3) the presence of yeast extract must be avoided; and (4) acetosyringone and a high sugar content must be present in the medium (see review by Hooykaas and Schilperpprt, 1992). Important variables related to co-cultivation also include the addition of acetosyringone to the medium and a temperature between 22 °C and 28 °C. Acetosyringone (AS) is a phenolic compound released by wounded cells from some of dicotyledonous plants (Stachel et al., 1985). It plays an important role in the natural infection of plants by *Agrobacterium* as it activates the virulence gene of the Ti plasmid and initiates the transfer of the T-DNA region into plant genome. Acetosyringone has been shown to increase Ti transformation efficiency (James et al., 1993; Joao and Brown, 1993; Sheikholeslam and Weeks, 1987). Acetosyringone was absolutely required in transformation of rice by *Agrobacterium* (Hiei et al.,1994). In the presence of AS, monosaccharide like D-glucose (Cangelosi et al., 1990; Khan et al.,1994: Shimoda et al., 1990) and osmoprotectants like betaine or proline (James et al., 1993; Vernade et al., 1988) can synergistically enhance *Agrobacterium* transformation.

1.1.2 Protoplast-mediated transformation

Direct DNA transfer, involving electroporation or chemical agents such as polyethlene glycol (PEG), requires the use of protoplasts. Both PEG and electroporation-mediated transformation involve causing reversible permeabilization of plasmic membranes that enable exogenous macromolecules to move into protoplasts (Jones et al., 1987; Krens et al., 1982; Zimmerman, 1986). Protoplasts, in principle, are ideal cells for DNA delivery and selection of transgenic events. Removal of the cell wall eliminates a major barrier to DNA delivery. Protoplast-derived cultures are generally clonal in origin (Songstad et al., 1995). Unfortunately, most crops including cereals proved to be very recalcitrant in regeneration from protoplasts, but through development of embryogenic suspension cultures as sources of totipotent cells (Vasil 1987), first rice and then maize, wheat, and other cereals have been regenerated from protoplasts with varying levels of success (Abduldahl et al., 1986; Fujimura et al., 1985; Rhodes et al., 1988a; Shillito et al.,1989). Almost at the same time the first transgenic rice (Toriyama et al., 1988; Shimamoto et al., 1989; Zhang and Wu, 1988) and maize (Rhodes et al., 1988b) plants were produced. But genotypic specificity still severely hinders transformation and subsequent regeneration of elite cultivars. For example, numerous reports of transgenic rice were limited to the *japonica* cv. Taipei 309. Most elite *japonica* as well as the vast *indica* and tropical *japonica* cultivars are very difficult to regenerate from protoplasts. Notable exceptions were described in the reports by Datta and his co-workers, which described recovery of fertile transgenic plants from cultivars Chinsurah Boro II (*indica* type, Datta et al., 1990) and IR72 (Datta et al., 1992). More recently, transgenic rice

plants with resistance to the sheath blight pathogen, *Rhizoctonia solani*, were produced from Chinsurah Boro II using the protoplast transformation approach (Lin et al., 1995). However, recent advances have seen the replacement of this approach by particle bombardment.

1.1.3 Particle bombardment technology

Particle bombardment utilizes high velocity metal particles to deliver biologically active DNA into plant cells. The technology was first reported by Klein et al. (1987). In their experiments, transient expression of exogenous RNA or DNA was demonstrated in the bombarded epidermal cells of onion (*Allium cepa*). The concept of particle bombardment (also known as biolistics, microprojectile bombardment, particle acceleration, etc.) has been described in detail by Sanford (1988). Following these experiments, the technique was shown to be the most versatile and effective way for the creation of transgenic organisms including microorganisms, mammalian cells and a large number of plant species (see review by Christou, 1995).

The ability to deliver foreign DNA into regenerable cells, tissues or organs, appeared to be the best method for achieving truly genotype-independent transformation in agronomic crops, bypassing *Agrobacterium* host-specificity and tissue culture-related regeneration difficulties. Due to the physical nature of the process, there is no biological constraint to the actual DNA delivery; thus genotype may not be a limiting factor. Combining the relative ease of DNA introduction into plant cells with efficient regeneration protocol for immature embryos and meristems, Christou et al. (1990, 1991)

established genotype-independent gene transfer methods using rice and soybean as model systems for monocotyledonous and dicotyledonous crops.

Several advantages made particle bombardment the method of choice for engineering crop species: 1) the ability to transform organized and potentially regenerable tissues, e.g., immature embryos for rice (Christou et al., 1991), meristems for cotton (McCabe and Martinell, 1993) and soybean (Christou et al.,1990) which allows introduction of foreign genes into elite germplasm; 2) universal delivery system: transient gene expression has been demonstrated in numerous tissues representing many different species; 3) transformation of recalcitrant species: engineering of important crops such as soybean, cotton, maize, rice, etc., is restricted in a few non-commercial varieties when conventional methods are used; particle bombardment technology allows recovery of transgenic plants from many commercial cultivars; 4) applicable to basic study of plant development: by utilizing chromogenic markers, it is possible to study many complex developmental processes(Christou, 1995).The disadvantages of particle bombardment include: 1) low consistency: the technology can be notorious for variability between results of consecutive bombardments under "identical" conditions; 2) the requirement of special equipment: a good system such as PDS/He1000 Biolistic system is costly and requires expensive gold powder as microprojectiles.

A number of parameters have been identified and need to be considered carefully in experiments using particle bombardment. The process involves a sequence of physical, chemical, and ballistic interactions involving DNA, high-velocity microprojectiles, and living target tissue. The following four key physical or chemical factors affect

transformation efficiency.1) Microprojectile composition and size: the microprojectiles must be available in a range of defined size ~1 μm diameter and of high density to achieve the momentum required for cell wall penetration. Microprojectiles must be chemically inert to prevent adverse reactions with DNA and toxicity to target cells. Suitable metal particles include gold and tungsten particles, etc. 2) DNA attachment to the microprojectiles: certain additives such as spermidine and calcium chloride proved essential for proper coating of metal particles with DNA (Klein et al., 1988). Recently, variations including PEG precipitation and $CaCl_2$/spermidine co-precipitation followed by an ethanol wash, has been used successfully (Christou et al., 1991; Ye et al., 1990). Improper or inconsistent DNA precipitation technique is a major cause of variation in gene transfer frequency. The DNA and gold particle load rates also affect transformation efficiency. 3) Microprojectile velocity: it is very important to deliver DNA into cells that are competent for both transformation and regeneration. The depth of penetration, determined by the microprojectile velocity, thus becomes one of the most important variables. Therefore, it is necessary to alter microprojectile velocity for optimal transformation rates with different tissue types, depending on cell wall thickness and the need to penetrate several cell layers. The velocity can be generally controlled to some degree by altering accelerating forces (i.e., gas pressure, electrical discharge energy, etc.), the vacuum in the target chamber, and the distance traveled by the microprojectiles. 4) The degree of tissue damage: in cases in which the old-type Biolistic gun is used, cells near the center of the target are injured and cannot proliferate. This injury was attributed to physical trauma of the cells from the gas blast and acoustic shock generated by the

device. The use of baffles with mesh screens reduced cell death and increased transformation frequency significantly (Gordon-Kamm et al., 1990; Russell et al., 1992a). Vacuum treatment to maintain projectile velocity may also affect plant development. Vacuum levels greater than 686 mm Hg are most effective to maintain velocity of particles around 1 μm in diameter (Klein et al., 1992). However, some plant tissues are sensitive to vacuum treatment. For example, the development of excised mature embryos of *Phaseolus vulgaris* cv. Sinatra is blocked after treatment with vacuum of 691 mm Hg, a level commonly used in particle bombardment. Also, increasing the number of bombardments increases the tissue damage, although multiple bombardments have resulted in an increase of transient gene expression (Talyer & Vasil, 1991). Hence, it is important to empirically determine the balance between excessive tissue damage and increased DNA transfer.

The biological parameters that affect the process include: 1) the nature of DNA: there are no special requirements for the form of DNA to be transferred. Some reports indicate increased transformation frequency when plasmid DNA is linearlized (Sautter et al., 1991), but others found no difference between linearlized and supercoiled plasmid (Klein et al., 1989). However, large plasmids (> 10 kb) may be more subject to fragmentation during bombardment, resulting in low expression and co-transformation frequency (Fitch et al., 1990; Mendel et al., 1989); 2) transgene construct: plant vectors used for free DNA delivery typically consist of an expression cassette that contains a promoter region, transcription initiation site, and a portion of the 5' nontranslated leader of the promoter of interest joined to a synthetic multilinker, followed by a

polyadenylation signal. Promoter strength is critical in allowing high-level transcription of selected sequences in plant cells. Although the 35S promoter from cauliflower mosaic virus (CaMV35S) has become the most widely used promoter in plant transformation, it is not regarded as the optimal promoter for monocots (Morrish et al., 1993). A change from CaMV 35S to a stronger monocot promoter, Emu, resulted in a 100-fold increase in transient gene expression in sugarcane (*Saccharum officinarum*) cell suspensions (Last et al., 1991). Maize ubiquitin promoter (Ubi1) directed significantly higher levels of transient gene expression in transgenic rice (Cornejo et al., 1993). Other promoters like maize alcohol dehydrogenase 1 (Adh1) promoter, a rice actin promoter (Act 1) (McElroy et al., 1990, and the rice ubiquitin promoter (Wang et al., unpublished) have also shown to enhance gene expression. Tissue-specific promoters are important because they allow targeting of gene expression to sites where genes are required. For example, a wheat glutenin promoter directed the endosperm-specific expression of high molecular weight glutenin (Thomas and Flavel, 1990). 3) target tissue characteristics: organized tissue with thicker cell walls require higher particle velocities for penetration than thin-walled cells from suspension culture. The frequency of expression of introduced DNA can vary dramatically between cell types. For example, all transiently expressing cells in bombarded sugarcane callus occurred in densely cytoplasmic, embryogenic callus regions, not in surrounding soft callus consisting of more vacuolated cells (Franks and Birch, 1991). Cells with large vacuoles may be more subject to damage by microprojectiles. Microscopic examination indicates that DNA-coated particles lodging in the nucleus have a transient expression frequency ~ 45 times higher than particles in

the cytosol and over 900 times higher than particles in the vacuole (Yamashita et al., 1991). Experiments performed with synchronized cultured cells indicates that transformation frequency is influenced by cell cycle stage (Iida et al., 1991). Transient expression frequencies declined sharply as wheat cell suspensions entered stationary phase (Vasil et al., 1991). Actively growing, healthy cells with reduced vacuole volumes, have been recommended for bombardment (Russell et al., 1992a). Osmotic treatment of target tissues significantly enhanced transformation efficiencies in tobacco (Russel et al., 1992b), maize (Vain et al., 1993), and rice (Zhang et al., 1996). The mechanism of osmotic enhancement could include both reduced vacuole volume and reduced turgor, which would alter cell penetrability by projectiles, and reduce cell damage by preventing extrusion of the protoplasm from bombarded cells. Choice of proper explants and pre- and post-bombardment culture conditions are factors that may determine whether experiment utilizing bombardment are successful. Temperature, photoperiod and humidity of environments under which donor plants, explants, and bombarded tissues are cultured are also important variables. Because such factors have a direct effect on the physiological state and receptiveness of target tissues to foreign DNA (Christou, 1995).

Despite the fact that numerous parameters influence particle bombardment-mediated transformation, the development of the bombardment approach has allowed the engineering of almost all major crops, including formerly recalcitrant cereals, legumes and woody species. Transgenic rice (Cao et al., 1992; Christou, 1991), wheat (Becker et al., 1994; Vasil et al., 1992; Weeks et al., 1993), maize (Fromm et al., 1990; Gordon-Kamm et al., 1990), barley (Wan and Lemaux, 1994), oat (*Avena sativa*) (Somers et al.,

1992), sorghum (*Sorgum bicolor*) (Casa et al., 1993), soybean (McCabe et al., 1988), sugarcane (Bower and Birch, 1992), sunflower (*Helianthus annuus*) (Bidney et al., 1992), canola (*Brassica napus*) (Chen and Beversdorf, 1994), poplar (*Populus spp.*) (Wilde et al., 1992), spruce (*Picea glauca*) (Ellis et al., 1993), cotton (Finer and McMullen, 1990; McCabe and Martinell, 1993) and others have been produced utilizing particle bombardment. More recently, many agronomically important genes have been introduced in rice employing particle bombardment. These include those genes conferring resistance to herbicides (Cao et al., 1992; Christou et al., 1991; Oard et al., 1996), viral diseases (Kloti et al., 1999; Zheng et al., 1997), fungal or bacterial diseases (Datta et al., 1999; Tang et al., 1999; Tu et al., 1998), insects (Duan et al., 1996; Nayak et al., 1997; Wu et al., 1997), and tolerance to water deficit and salt stress (Xu et al., 1996).

Like other methods, the bombardment approach has its drawbacks. In some cases, the copy number and rearrangement of the introduced DNA is high, thereby rendering transgene prone to gene silencing (Kumpatala et al., 1997) and genomic changes. However, recent data do not support the fears about gene silencing and genetic integrity (Arencibia et al., 1998). Kohli et al. (1998) maintained that transformation through direct DNA transfer, specifically particle bombardment, generally results in a single transgenic locus as a result of a two-phase integration mechanism, thereby avoiding the problem of segregation of transgenes during breeding programs. Kohli et al. (1999) further observed that an increase in transgene copy number did not always lead to a concomitant decrease in expression levels or to silencing through co-suppression. They found transgenic lines

with four to five copies of integrated transgene expressed the protein product of transgenes stably and at levels comparable to transformants with one or two copies. The particle bombardment approach seems to be the most versatile and successful approach for transformation of cereal crops (see Christou, 1997). This will certainly be the case until *Agrobacterium*-mediated transformation in monocots including rice, which has seen some success in recent years, becomes routine and "super-virulent" *Agrobacterium* stains harboring agronomically useful genes are constructed.

1.2 Engineering Herbicide Resistance in Plants

The use of herbicides to reduce loss in crop yield due to weeds has become an important component of modern agriculture. An ideal herbicide should combine the following properties: 1) control of all plant species except for the crop of interest; 2) high activity; 3) environmentally friendly: safe for men and animals, no soil residue or minimal persistence in soil. However, it is difficult to find a herbicide with all these properties. Most herbicides are designed to affect the photosynthetic process or inhibit specific amino acid biosynthesis pathways in plants (La Rossa and Falco,1984). Since photosynthesis and the enzymes in amino acid biosynthesis that herbicides inhibit are common to all plants, weeds as well as crop plants, most herbicides can not be sprayed for post-emergence control. Development of herbicide resistance in crop plants using genetic engineering was one of the first applications of transgenic technology to a commercially important objective. Two main strategies have been followed to obtain herbicide-resistant plants. 1) decreasing the sensitivity of the plant to the herbicide by

overproduction of target enzyme or by expression a modified target which is insensitive to the herbicide but retaining enzymatic activity, and 2) providing a herbicide-detoxifying enzyme to plants.

1.2.1 Altering the level and sensitivity of target enzymes of herbicides

An example of this approach is engineered resistance to glyphosate. Glyphosate (marketed as Roundup®, by Monsanto) is a potent broad-spectrum non-selective herbicide. It is an inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), an enzyme in the biosynthetic pathway of three aromatic amino acids, phenylalanine, tyrosine and tryptophan. The introduction of mutant *EPSPS* genes, encoding a structurally altered enzymes less sensitive to glyphosate (Comai et al., 1985; Fillatti et al., 1987) or rendering the overproduction of plant *EPSPS* (Shah et al., 1986b), conferred tolerance to glyphosate in transgenic plants. The resistance to Roundup Ultra in Roundup Ready® soybeans is the result of expression of a transgene conferring CP4-EPSPS, a Roundup-tolerant enzyme. Similarly, transgenic tobacco plants expressing mutant acetolactate synthase (ALS) were found to be tolerant to sulphonylurea herbicide compounds (Haughn et al., 1988; Lee et al., 1988).

1.2.2 Transferring herbicide-detoxifying genes

High levels of resistance towards several herbicides were obtained with the second approach, i.e. engineering of a novel detoxification pathway in plants. Herbicide-detoxifying enzymes have been identified in several plant species and in microorganisms and were exploited to engineer herbicide resistance in plants.

1.2.2.1 Herbicide-detoxifying genes from plants

Little information is available on the molecular basis of herbicide detoxification in plants. Consequently, the development of a genetic engineering strategy based on plant herbicide-detoxifying enzymes is slower than that of microbial systems. Herbicide-detoxifying enzymes such as mixed function oxidase, amidase, decarboxylase and conjugative systems have been identified in tolerant plants (Donn et al., 1984). Genes encoding atrazine and alachlor detoxifying enzymes, glutathione-S-transferase (GSTs), have been isolated from maize. However, the introduction and expression in other plants has met little success (Shah et al., 1986a). Another class of a conjugative enzyme is represented by the N-glucosyl transferase present in metribuzin-tolerant tomato (Comai and Stakler, 1986). Detoxication of the herbicide is due to increased activity of N-glucoyl transferase. A third class of plant enzyme is the mixed function oxidase, involved in the detoxification of 2,4-D in tolerant pea and of dicamba in tolerant barley (Comai and Stakler, 1986).

1.2.2.2 Herbicide-detoxifying genes from bacteria

The introduction of herbicide-detoxifying genes from bacteria into plants was an successful strategy used in development of resistance to three different herbicides: bromoxynil, phosphinothricin (glufosinate), and 2,4-dichlorophenoxyacetic acid (2,4-D). Bromoxynil (Buctril) is a potent inhibitor of photosystem II with good activity in dicotyledonous plants. The biochemical target of bromoxynil is known to be located in the chloroplast. Transgenic tobacco plants resistant to high doses of a commercial formulation of bromoxynil were generated by transformation of a gene from *Klebsiella*

ozaenae (*bxn*), which encodes a specific nitrilase that converts the cyano moiety of bromoxynil to the non-phytotoxic acid derivative (Stalker et al., 1988). The bacterial gene was driven by a promoter derived from a tobacco light-inducible, tissue-specific ribulose biphosphate carboxylase small subunit gene to restrict expression to photosynthetic tissues. The commercially available BXN transgenic cotton was developed by the same strategy. Similarly, engineered resistance to 2,4-D in transgenic tobacco plants was achieved by the introduction of a bacterial gene (*tfd*A) encoding 2,4-D monooxygenase via *Agrobacterium* (Lyon et al., 1989). 2,4-D monooxygenase catalyzes the cleavage of an acetate side chain of 2,4-D to give glyoxylate and 2,4-dichlorophenol which is between 50 and 100 times less toxic to tobacco plants (Streber and Willmitzer, 1989).

De Block et al. (1987) reported engineering of plants resistant to two nonselective herbicides, phosphinothricin (PPT) and bialaphos. Bialaphos is a tripeptide antibiotic which consists of PPT, an analogue of L-glutamic acid, and two L- alanine residues. PPT is a potent inhibitor of glutamine synthetase (GS) which plays a central role in the assimilation of ammonia and the regulation of nitrogen metabolism in plants. It is the only enzyme in plants that detoxifies ammonia released by nitrate reduction, amino acid degradation, and photorespiration. Inhibition of GS by PPT causes rapid accumulation of ammonia which leads to death of the plant cells (Tachibana et al., 1986). At present, two products are available as commercial herbicides: glufosinate ammonium is the ammonium salt of the chemically synthesized PPT (Basta, Hoechst AG, FRG), and bialaphos is produced by fermentation of *S. hygroscopicus* (Herviace. Meiji Seika Ltd). A PPT resistance gene, *bar*, was isolated from *S. hygroscopicus* (Murakami et al., 1986;

Thompson et al., 1987) and was shown to encode the enzyme phosphinothricin acetyltransferase (PAT), which specifically coverts PPT to an acetylated, non-herbicidal form (Thompson et al., 1987).

The *bar* gene under the control of the CaMV35 promoter was introduced into tobacco, tomato, potato, oilseed rape (*Brassica napus*), alfalfa (*Medicago sativa*), sugarbeet (*Beta vulgaris*), aspen (*Populus tremuloides*) and poplar plants via *Agrobacterium* (Botterman and Leemans, 1988; De Block et al.,1987, 1989; D'Halluin et al., 1990). In all cases, the *bar* gene conferred resistance in greenhouse-grown transgenic plants to doses of glufosinate and bialaphos which were higher than normally applied in agriculture. Field tests with transgenic tobacco, potato and alfalfa plants, complete resistance was observed (De Greef et al., 1989; D'Halluin et al., 1990). Furthermore, the herbicide-treated transgenic plants revealed the same agronomic performance as non-transformed, non-treated control plants (De Greef et al., 1989). More recently, the *bar* gene has been introduced into rice (Cao et al., 1992; Christou et al., 1991, 1992, 1995; Datta et al., 1992), maize (Gordon-Kamm et al., 1990), wheat (Vasil et al., 1992, 1993; Weeks et al., 1993), barley (Stiff et al., 1995; Wan and Lemaux, 1994) and soybean (Christou et al., 1990), primarily by biolistic particle bombardment. Resistance to glufosinate ammonium has been demonstrated in transgenic elite rice in the field trials performed at the LSU Rice Research Station at Crowley, LA since 1993 (Christou,1995; Oard et al., 1996). Similar results was observed in herbicide-resistance soybean field trials (Christou, 1995). All these results convincingly demonstrated that the broad-

spectrum herbicide glufosinate can be used selectively for post-emergence application on transgenic crop plants.

Another PPT-resistant gene, *pat*, was isolated and cloned from *Streptomyces viridochromogenes* Tu494 (Strauch et al., 1988; Wohlleben et al.,1988). The *pat* gene also encodes a phosphinothricin-N-acetyl-transferase (PAT), which inactivates PPT by acetylation. The bacterial pat gene was modified to enable its expression in plants. The GTG start codon of *pat* was replaced by ATG. The modified *pat* gene has been introduced via *Agrobacterium*-mediated gene transfer into *Nicotiana tobacum* (Wohlleben et al., 1988; Droge et al., 1992), via direct gene transfer into carrot (*Daucus carota*) (Droge et al., 1992). A *pat*-based synthetic gene has also been used to transform alfalfa (*Medicago sativa*) (Eckes et al., 1989), and tobacco (Hoeven et al., 1994).

Wohllebed et al. (1988) compared the sequence of the *bar* gene from *S. hygroscopicus* (Thompson et al. 1987) with the *pat* gene of *S. viridochromogenes* and found significant homology between the two genes. However, variation was detected in the 5' noncoding region of the two resistance genes which may reflect differences in regulation. Introduction of the *pat* gene into cereal crops including rice may lead to more herbicide-resistant crops and extend the agricultural application of glufosinate.

1.2.3 Conclusions

The development of herbicide-resistant crop cultivars has generated not only considerable interest, but also considerable controversy over recent years. A number of genetically engineered herbicide-resistant crops, such as BXN cotton, Roundup-Ready® soybean, Liberty Link® corn have been released for commercial production since1994.

Field trials of Liberty Link® rice and Roundup Ready® rice are ongoing. The availability of transgenic technology will add to the arsenal of integrated pest management options available to the growers (Griffin and Micinski, 1998). However, there is a fear of an environmental risk that the transgenic crop that is herbicide-resistant may become a volunteer weed, i.e., unharvested, shattered seeds of the crop germinating in the following crop, where it is, by definition, a weed (Gressal, 1993). The problem will be difficult to control. Another factor that needs to be considered is the likelihood of the herbicide-resistant genes becoming established in weed populations by hybridization between crop and weeds. In general, the herbicide resistance is controlled by a dominant single gene (Mazur and Falco, 1989). If the hybrids are fertile, they may be difficult to control in an agricultural system which depends on weed control with the same herbicide (Dale and Kinderlerer, 1995). It has been found that in rice fields, red rice (also belonging to *Oryza sativa*), a noxious weed, can be hybridized with commercial rice (Dr. Steve Linscombe, personal communications). Risk assessments should be made to identify and prevent the possible consequences of such a gene transfer (Dale and Kinderlerer, 1995).

CHAPTER 2. HIGH EFFICIENCY TRANSFORMATION OF U.S. RICE LINES FROM MATURE SEED-DERIVED CALLI AND SEGREGATION OF GLUFOSINATE RESISTANCE UNDER FIELD CONDITIONS

2.1 Introduction

Significant advances have been made in the development of rice (*Oryza sativa*) genetic transformation methods and incorporation of genes conferring important agronomic traits in the last decade. The first transgenic rice plants were obtained from protoplast transformation systems with DNA uptake mediated by electroporation (Shimamoto et al., 1989; Toriyama et al., 1988; Zhang et al., 1988) or polyethylene glycol (PEG) (Datta et al., 1990; Zhang and Wu, 1988). However, since this method is limited by constraints imposed by delicate protoplast culture systems, only a few *japonica* and even a smaller number of *indica* cultivars could be engineered for resistance to glufosinate herbicide (Datta et al., 1992), rice striped stem borer (*Chilo suppressalis*), rice leaf folder (*Cnaphalocrocis medinalis*) (Fujimoto et al., 1993), and the sheath blight pathogen, *Rhizoctonia solani* (Lin et al.,1995). With the development of bombardment-based methodology (Klein et al., 1987; Sanford, 1988), production of transgenic rice plants from elite *indica* and *japonica* rice cultivars by particle bombardment has been reported by different laboratories (Abedinia et al., 1997; Cao et al., 1992; Christou et al., 1991; Ghosh Biswas et al., 1998; Li et al., 1993 Nayak et al., 1997; Sivamani et al., 1996; Valdez et al., 1998; Zhang et al., 1996). Transgenic rice plants expressing resistance to glufosinate (Cao et al., 1992; Christou et al., 1991; Cooley et al., 1995), the bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae* race 6 (Song et al.,1995; Tu et al.,1998;

Tang et al., 1999), yellow stem borer (*Scirpophaga incertulas*) (Datta et al., 1998; Maqbool et al., 1998; Nayak et al, 1997; Wu et al., 1998), leaf folder (*Cnaphalocrocis medinalis*) (Maqbool et al., 1998), pink stem borer (*Sesamia inferens*) (Duan et al., 1996), nematode (*Meloidogyne incognita*) (Vain et al., 1998), and tolerance to water deficit and salt stress (Xu et al., 1996) have been obtained via particle bombardment. The first successful rice field tests of transgenic glufosinate-resistant rice was reported by Oard et al., 1996. However, hygromycin was the only selection agent used to identify transformants from immature embryo-derived resistant calli. Moreover, segregation analysis for glufosinate resistance was not performed. Substantial differences were observed in terms of genotype, type of explant, selection agent, and transformation efficiency, subject to the objective of each experiment. Due to their ideal responsiveness in tissue culture and higher regeneration potential, immature embryo explants were first used in recovery of transgenic elite *indica* and *japonica* rice (Christou et al., 1991; Cooley et al., 1995; Li et al., 1993; Vain et al., 1998). However, preparation of a large number of high quality immature embryos was labor intensive, and environment- and season-dependent, which made the protocol difficult to adapt in other laboratories. Because of convenience in preparing target tissues and good receptiveness for high transformation efficiency with bombardment, embryogenic suspension culture cells were used for production of transgenic rice in the model *japonica* genotypes Taipei 309 (Cao et al., 1992), Nipponbare, Tainung 67, and Pi4 (Duan et al., 1996), elite *indica* (group 1) rice cultivars IR24, IR64, IR72 (Zhang et al., 1996) and *indica* Basmatic rice cultivar Pusa Basmati 1 (Jain et al., 1996). Nevertheless, there is still a drawback in this approach

because establishment of embryogenic suspension cell lines is time-consuming and can be difficult for some commercial genotypes. Furthermore, long-term maintenance of suspension cells can result in unexpected somaclonal variation in the target cells prior to transformation.

To circumvent problems and disadvantages of using immature embryos or suspension cells as target tissues for bombardment, Sivamani et al.1996 reported selection of large quantities of embryogenic calli from rice seeds as target tissues and use of hygromycin B as an effective selection agent to produce fertile transgenic rice plants in the model *indica* cv. TN 1. Using basically the same or slightly modified protocol as described by Sivamani et al. 1996, fertile transgenic rice plants were obtained for an Australian rice cultivar Jarrah (Abedinia et al., 1997), Taipei 309 (Chen et al., 1998), and diverse tropical *japonica* and *indica* cultivars (Ghosh Biswas et al., 1998). Valdez et al. (1998) reported a novel system by which transgenic rice plants were produced by direct bombardment of mature embryos instead of mature embryo-derived embryogenic calli. However, no detailed data were provided to demonstrate its transformation efficiency. Therefore, it remains a challenge to efficiently develop transgenic rice plants from mature seed embryos for elite tropical *japonica* U.S. rice breeding lines, including long-grain commercial cultivars, especially when selection of transformants with hygromycin is not desirable.

The objectives of the present study are: 1) to develop a routine and efficient transformation system for elite U.S. rice lines by particle bombardment of mature seed-derived calli, independent of season and environment; and 2) to produce a large number

of elite glufosinate-resistant transgenic lines and study segregation of glufosinate resistance and expression of transgenes under field conditions.

2.2 Materials and Methods

2.2.1 Plant materials and *in vitro* callus production

One commercial rice cultivar, Cocodrie, and 5 elite U.S. breeding lines LA9502065, LA9502002, 96URN082, 96URN085, and 96URN131 were used in the transformation experiments (Table 2.3). Mature seeds were dehusked and surface-sterilized in 50% (v/v) commercial bleach (containing 6.25 % sodium hypochlorite) for 45 min followed by 3 rinses in sterile distilled water. Seeds were aseptically plated on CI medium (Table 2.1) for callus induction. After 3 weeks in the dark at 26 °C, embryogenic calli initiated on the scutellar surface of mature seed embryos were selected and subcultured at three weeks intervals on the same medium.

2.2.2 Plasmids used and constructed for bombardment studies

Four DNA plasmids were used for bombardment experiments: pAHC25 (Christensen et al., 1992), pTRA151 (Zheng et al.,1991), pB2/35SAck (Hoechst Schering AgrEvo), and pPAT63 (this study) (Figure 2.1). pAHC25 consists of the *uidA* gene encoding β-glucuronidase (GUS) (Jefferson, 1987) and the *bar* gene (Thompson et al., 1987) encoding phosphinothricin acetyltransferase (PAT) that inactivates phosphinothricin (PPT), active ingredient of the herbicide glufosinate (trade names Liberty, Basta, Ignite, Finale). Transcription of *uid*A and *bar* genes were under the control of the maize ubiquitin *Ubi1* promoter (Christensen et al., 1992). The plasmid

pB2/35SAcK, 4.1 kb in size with a pUC19 backbone, contains a synthetic *pat* gene (Eckes et al., 1989) encoding the PAT enzyme fused to a 35S-promoter and 35-terminator. To facilitate selection of transformants with hygromycin B, a 1.7 kb *Hin*d III fragment containing a mutated version of the *hph* gene from plasmid pTRA151 (Zheng et al., 1991) was cloned into a unique *Hin*d III site of pB2/35AcK by the author. The resulting plasmid was designated pPAT63.

2.2.3 Particle bombardment

The helium-driven Biolistic PDS-1000/He (Bio-Rad) system was used in all experiments. Plasmid DNAs were coated onto gold particles (1 μm in size) according to Sanford et al.(1992). The DNA coating protocol described by Weeks et al. (1993) was also evaluated in a pilot test. Approximately 30 embryogenic calli (2-3 mm in diameter) were placed evenly onto a 100 x 15 mm plastic Petri dish containing 25 ml of fresh CI medium one day prior to bombardment. For routine bombardment, the distance from rupture disc to macro-carrier, from macro-carrier to stopping screen, and from stopping screen to target tissues was 1, 1, and 9 cm, respectively. The rupture disc strength was 1,100 psi and vacuum pressure of the bombardment chamber was 0.09 MPa (27 in Hg). Bombardment was conducted once per petri dish.

2.2.4 GUS assays

To optimize bombardment parameters and establish a guideline for stable transformation for glufosinate resistance, GUS activity was assayed histochemically two

Table 2.1. Composition of media for rice tissue culture and transformation experiments in this study.

Medium	Composition
CI	CC salts and vitamins (Potrykus et al. 1979), 20 g L^{-1} sucrose, 18.2 g L^{-1} mannitol, 18.2 g L^{-1} sorbitol, 2 mg L^{-1} 2,4-D, 7 g L^{-1} agarose (Type I-A, Sigma), pH 5.8
CI-H25	CI with 25 mg L^{-1} hygromycin B (Boehringer Mannheim)
CI-H50	CI with 50 mg L^{-1} hygromycin B
CI-B2	CI with 2 mg L^{-1} bialaphos (Meiji Seika Kaisha, Yokyo, Japan)
CI-B4	CI with 4 mg L^{-1} bialaphos
CI-B10	CI with 10 mg L^{-1} bialaphos
PR1	CC salts and vitamin (Potrykus et al. 1979), 1.0 g L^{-1} casein hydrolysate, 20 g L^{-1} sucrose, 36.4 g L^{-1} sorbitol, 1 mg L^{-1} IAA, 0.05 mg L^{-1} zeatin ribosome, 7 g L^{-1} agarose (Type I-A, Sigma), pH 5.8
PR1-H50	PR1 with 50 mg L^{-1} hygromycin B
PR1-B4	PR1 with 4 mg L^{-1} bialaphos
PR2	MS salts and vitamins (Murashige and Skoog,1962), 20 g L^{-1} sucrose, 10 g L^{-1} maltose, 2 mg L^{-1} kinetin, 0.5 mg L^{-1} NAA, 7 g L^{-1} agarose (Type I-A, Sigma), pH 5.8
PR2-H50	PR2 with 50 mg L^{-1} hygromycin
PR2-B4	PR2 with 4 mg L^{-1} bialaphos
RT	MS salts and vitamins (Murashige and Skoog, 1962), 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel (Sigma), pH 5.8
RT-B4	RT with 4 mg L^{-1} bialaphos

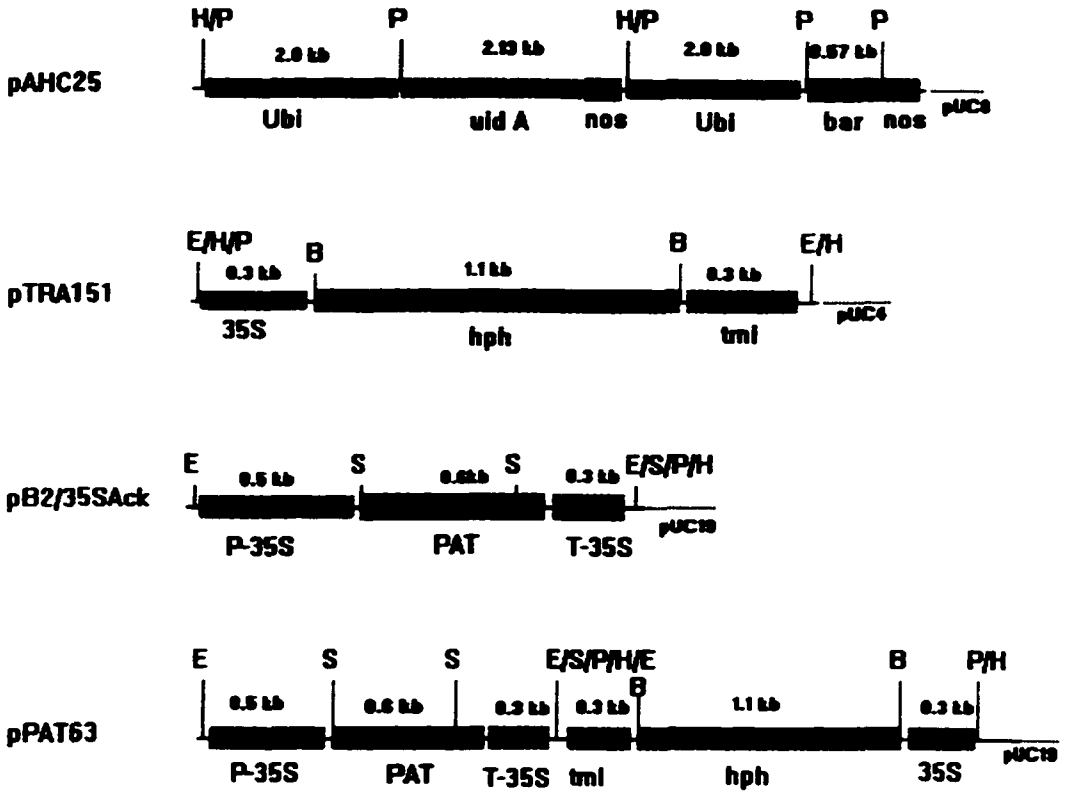

Fig.2.1. Schematic representation of plasmid constructs used in this study. Ubi, maize ubiquitin promotor; nos, terminator of nopaline synthetase; 35S or P-35S, 35 S promotor of cauliflower mosaic virus; T-35S, 35S terminator of cauliflower mosaic virus; tml, transcription terminator of a tumor morphology large gene. H, *Hind* III; P, *Pst* I; E, *Eco*R I; B, *Bam*H I; S, *Sma*I.

days after bombardment as described by Jefferson et al. (1987). Bombarded calli were incubated in phosphate buffer (100 mM $NaPO_3$, pH 7.0), that contained 1.0 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), 1% Triton X-100 at 37 °C, for 4 hours. Presence of any blue spots in a callus indicated transient expression of the *uidA* (GUS) gene.

2.2.5 Selection of transformants and regeneration of putative transgenic plants

One day after bombardment, embryogenic calli were transferred to CI medium supplemented with hygromycin B (Boehringer Mannheim) or bialaphos (Meiji Seika Kaisha, Tokyo, Japan) in different concentrations (Table 2.2). Calli were subcultured on the same fresh selection medium at 3-week intervals. After approximately 5 to 8 wks of selection, 2 to 3 mm in diameter, hygromycin- or bialaphos-resistant calli were recovered and transferred to PR1 or PR2 regeneration medium (Table 2.1). In approximately 3 wks, shoots or plantlets were recovered and transferred into Magenta boxes (Sigma) containing RT medium (Table 2.1). Plantlets 7-10 cm high with vigorous root development were transferred to potting soil in the greenhouse and designated as R0 plants.

2.2.6 Glufosinate herbicide application in the greenhouse

Putative transgenic rice plants grown to the third- or fourth leaf stage in the greenhouse were tested for response to Glufosinate herbicide (Liberty, 200 g L^{-1} PPT, Hoechst AG, Germany) by spraying with a 0.25 % to 1.0 % (v/v) solution plus 0.1 % (v/v) Tween 20. The actual concentration of PPT used was 500 to 2000 mg L^{-1}. Resistant and susceptible plants were scored 7 DAT.

2.2.7 Southern hybridization analysis

Genomic DNAs were extracted from leaf tissues according to Dellaporta et al. (1984). Purified DNAs for each sample, undigested or digested with proper restriction enzymes, were electrophoresed in 1.0 % agarose gels, transferred onto Hybond-NX (Amersham) membrane , and fixed using a UV Crosslinker (FB-UVXL-1000, FisherBiotech) set to deliver an energy dosage of 700 J m^{-2} as recommended by the manufacturer. Membranes were prehybridized at 65 °C for 2 hours in a buffer containing 6x SSC, 0.5% (w/v) SDS, 5 x Denhardt's solution, and 100 mg L^{-1} sheared, denatured salmon sperm DNA, and then hybridized at 65 °C overnight in the same buffer containing probes labeled with [α-^{32}P] dCTP (3,000 Ci/mol, Amersham) using random primed DNA labeling procedures (Feinberg and Vogelstein, 1983 ; Boehringer-Mannheim). A 0.45 kb *Sma* I fragment from pPAT63 and a 1.1 kb *BamH* I fragment from pTRA151 (Fig. 2.1) were used as probes for the *pat* and *hph* genes, respectively. After hybridization, membranes were washed (Sambrook et al.,1989) and exposed to Kodak BioMax MS autoradiography films with a Kodak BioMax MS intensifying screen at -80 °C for 16 hours. Membranes were stripped for re-hybridization by washing twice, for 15 min each wash, in 0.1% SSC at 100 °C.

2.2.8 Germination test of mature R1 seeds

Seeds of self-pollinated R0 transgenic plants were designated as R1 seeds. In the first experiments, transmission of the *pat* and *bar* gene was examined by germination of R1 seeds on RT-B4 medium containing 4 mg L^{-1} bialaphos (Table 2.1). The number of germinated seeds was determined after one week. Seedlings from germinated seeds were

transferred to the greenhouse and tested further for response to glufosinate by spraying to runoff with a 1.0 % (v/v) aqueous solution.

2.2.9 Segregation of glufosinate resistance in R1 and R2 progeny

To evaluate R1 transmission of the *pat* or *bar* gene in the field, R1 seeds from 20 independently transformed lines of Cocodrie (Table 2.4 and 2.5) and 10 independently transformed lines of LA9502065 (Table 2.6) were directly germinated in soil in the greenhouse. Seedlings at the 2-3 leaf stage were transplanted June1998 to the field at the LSU Rice Research Station, Crowley, LA. At the third to fourth leaf stage, plants were sprayed with glufosinate herbicide at a rate of 1.12 kg ha^{-1}. Resistant and susceptible plants were counted one week after treatment. Seeds from glufosinate-resistant, self-pollinated R1 plants were designated as R2 seeds. Glufosinate resistance in the R2 progeny was tested both in the greenhouse during the winter of 1998 and in the field at Lajas, Puerto Rico during spring 1999 in the same manner as described above.

2.3 Results and Discussion

2.3.1 Preparation of embryogenic calli from mature seed embryos for transformation and regeneration

Selection of target tissues is a critical factor when developing transgenic rice by bombardment. The primary requirement for an optimal target is that tissues or cells receiving exogenous DNA are culturable *in vitro*, actively dividing, and capable of regenerating into fertile plants. In previous rice transformation studies, it was reported that immature embryos (Christou et al., 1991; Cooley et al., 1995; Li et al., 1993),

embryogenic calli derived from immature embryos (Li et al., 1993), and embryogenic cell suspensions (Jain et al., 1996; Zhang et al., 1996) were competent for transformation and regeneration. However, preparation of a large quantity of immature embryos or embryogenic cell suspensions is labor extensive and environment- or genotype-dependent, whereas mature seed embryos are available year round. Therefore, mature seed embryo-derived embryogenic calli was considered by the authors as suitable target tissues for bombardment transformation of elite U.S. rice breeding lines. While this research was being conducted, Sivamani et al. (1996) reported production of fertile transgenic plants from embryogenic calli derived from seeds of the *indica* cultivar TN1. The same group recently described a protocol for consistent, large scale production of fertile transgenic rice plants in the *japonica* model cultivar Taipei 309 (Chen et al., 1998). However, the applicability of their protocol to a wide range of genotypes has not been demonstrated. This is an essential prerequisite for transfer of agronomically important genes into various elite U.S. cultivars and breeding lines.

For efficient induction of embryogenic calli from mature seeds of elite U.S. breeding lines, five different basal media, i.e., MS (Murashige and Skoog, 1962), N6 (Chu et al., 1975), NB (Sivamani et al., 1996), LS (Linsmaier and Skoog, 1965), and CI, a modified CC medium (Potrykus et al., 1979) were tested for their efficiency. Our results indicated that CI was the best medium for induction and proliferation of embryogenic calli from mature seed embryos in elite long-grain U.S. rice lines including the newest cultivar Cocodrie. Primary embryogenic calli induced from mature seeds (Fig. 2.2a) were

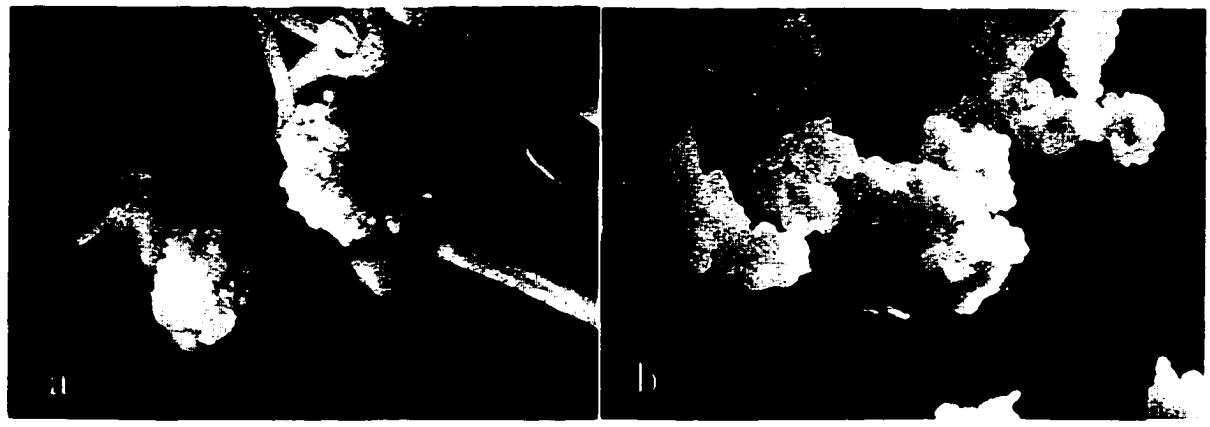

Fig 2.2. Preparation of embryogenic calli from mature seeds of rice cultivar Cocodrie for transformation. (a) Somatic embryogenic calli induced from mature seeds in CI medium. (b) Proliferation of embryogenic calli in subculture CI medium. x5.

selected and subcultured every 2 to 3 wks in fresh CI medium for proliferation. Within 3-4 months, a large quantity of independent embryogenic calli (Fig. 2.2b) was obtained. When these calli were transferred onto PR-1 or PR-2 regeneration medium, shoots and roots developed within 3 wks. A series of regeneration experiments revealed that the regeneration potential of embryogenic calli could be maintained at least for 4 to 5 months (data not shown). By this means, a large quantity of optimal target tissues was prepared for bombardment.

2.3.2 Transient expression and optimization of transformation

Bombardment parameters were optimized to increase the frequency of transformation events using pAHC25. Parameters influencing the delivery of DNA-coated gold particles included DNA coating procedure, the pressure strength of rupture discs, the distance between the stopping screen and the target tissues, and number of

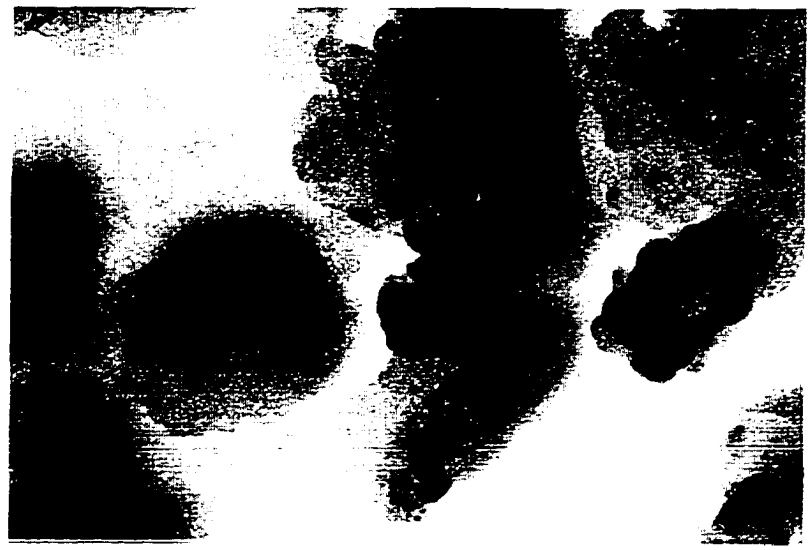

Fig. 2.3. GUS gene expression in embryogenic calli 2 days after bombardment with plasmid pAHC25. The calli were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). The dark blue spots in the color print represented transient expression of GUS (*uidA*) gene. x20.

bombardment. DNA coating protocols basically as described by Sanford et al. (1992) and Weeks et al. (1993) were compared, both rendering high levels (approximately 2,000 blue spots per plate) of GUS expression. However, uneven aggregation of particles on the surface of target cells and browning of cells was observed with the protocol of Weeks et al. (1993), probably due to the larger amount of gold particles per bombardment. Based on GUS expression, the distances of 9 cm and 13 cm between the stopping screen and the target tissues were found to be optimal. In previous reports target tissues were bombarded twice (Chen et al., 1998; Jain et al., 1996; Li et al., 1993), whereas in all of our bombardment experiments only a single bombardment was performed that reproducibly yielded high levels of GUS expression (Fig 2.3) as well as stable transformation. This

simplified the bombardment procedure and reduced the possibility of physically damaging the living cells of target tissues by two or multiple bombardments.

2.3.3 Selection of stable transformants using hygromycin and bialaphos

Production of numerous independently transformed calli and fertile transgenic rice plants was the result of using only proliferated embryogenic calli as target tissues and the execution of stringent selection. In the initial attempt to develop transgenic rice plants from LA9502065, a total of 590 embryogenic calli bombarded with pPAT63 were immediately transferred onto regeneration medium in the absence of any selection agent. A total of 798 plants were obtained with an optimal regeneration system. To recover stable transformants, all regenerated plants were transplanted in the greenhouse and were sprayed with 2000 mg L^{-1} glufosinate at the 4 to 5 leaf stage. No plants survived (Table 2.2), indicating that all regenerated plants were produced from non-transformed cells. The negative result indicated that a rice transformation protocol without selection is labor-intensive and fruitless. This result was inconsistent with previous reports in rice (Christou et al., 1991) and barley (*Hordeum vulgare*) (Ritala et al., 1994), where transformed embryogenic calli and somatic embryos were recovered in the absence of any selection pressure.

To establish an efficient transformation system, different selection methods with the antibiotic hygromycin B at two concentrations (25, 50 mg L^{-1}) and the herbicide bialaphos at three levels (2, 4, and 10 mg L^{-1}) were evaluated in the recovery of the stable transformants yielding glufosinate resistant plants (Table 2.2). The selection process

Table 2.2. Transformation efficiency of glufosinate resistant lines of LA9502065 using hygromycin B or bialaphos as a selection agent.

Selection agent	Conc. (mg L^{-1})	No. of calli bombarded (A)	No. of resistant calli	No. of plants regenerated (B)	No. of glufosinate resistant plants (C)	% of glufosinate resistant plants (C/B)	Transform-ation efficiency (C/A)
None	0	590	-	798	0	0	0
Hygromycin B	25	250	27	43	6	14	0.024
	50	250	12	56	54	96	0.216
Bialaphos	2	250	13	14	2	14	0.008
	4	250	9	33	33	100	0.132
	10	250	7	45	45	100	0.180

included three stages: 1) recovery of antibiotic- or herbicide-resistant calli among bombarded target tissues (Fig. 2.4a); 2) plant regeneration from antibiotic- or bialaphos-resistant calli in the presence of the selective agent (Fig. 2.4b); 3) induction of roots of regenerated plantlets in the continuous presence of the selective agent. The regenerated plants were then grown to the 3-4 leaf stage and tested for glufosinate resistance in the greenhouse (Fig. 2.4c). The test for herbicide glufosinate resistance in rice plants was quick and effective and distinguished transformants from non-transformants including escapes. Two days after spraying glufosinate, chlorotic leaves were observed in untransformed plants while transgenic rice plants exhibited no chlorosis. Approximately 6 days after glufosinate application, untransformed plants were killed while transgenic plants survived, showing total resistance or tolerance to the herbicide (Fig. 2.4d). The

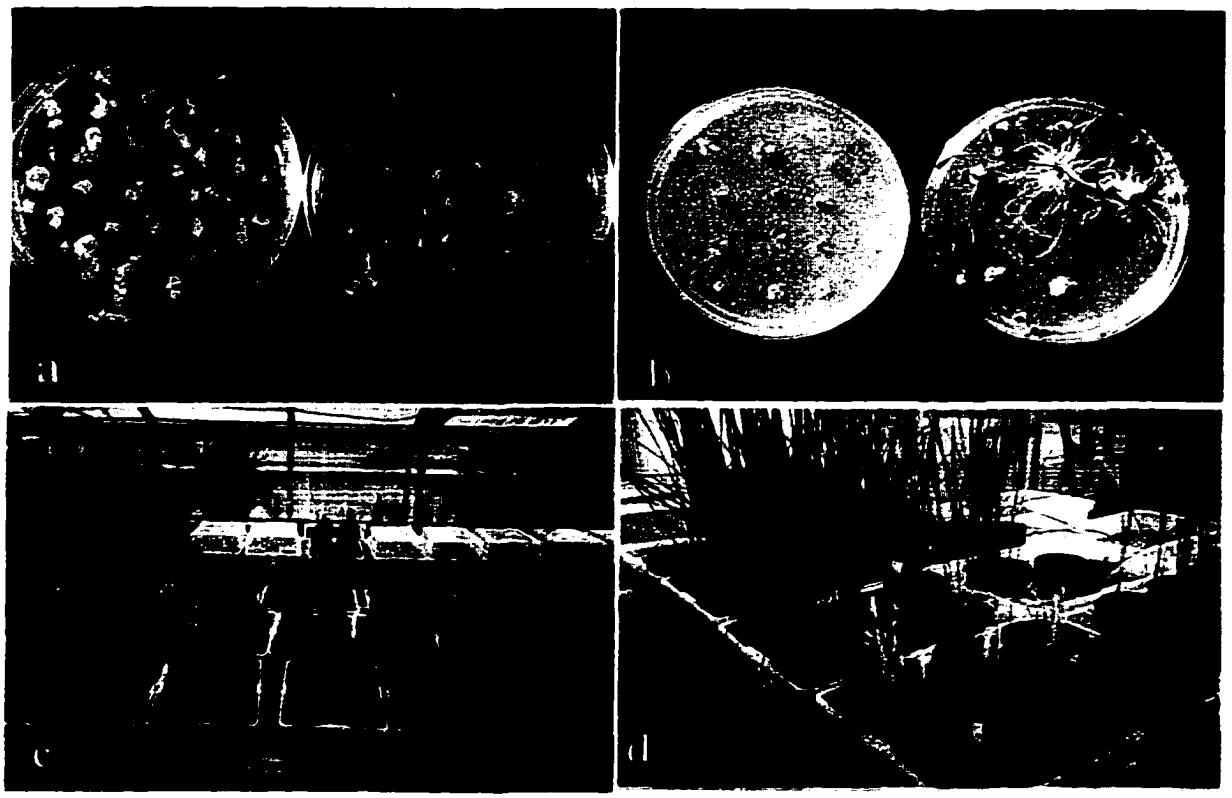

Fig. 2.4. Production and greenhouse tests of transgenic rice plants for glufosinate herbicide resistance. (a) Growth of bombarded calli in the absence (left) vs. presence (right) of bialaphos, transformed cells were recovered in the presence of 4.0 mg/L bialaphos. (b) Plantlet regeneration (right plate) of resistant calli in PR-2 medium containing 4 mg/L bialaphos. (c) Plantlets regenerated from transformed and untransformed cells were transferred and grown in the greenhouse. (d) Transgenic rice (R0) plants (background) survived while untransformed rice were killed (foreground) 7 days after foliar application of glufosinate at 2000 mg L^{-1}.

results showed that directly using bialaphos at concentrations of 4 and 10 mg L^{-1} as a selective agent, all regenerated plants were resistant to glufosinate with a transformation efficiency of 13% and 18%, respectively. However, when the bialaphos concentration

was as low as 2 mg L^{-1}, transformation efficiency was reduced to 0.8 %, indicating that the low selection pressure resulted in many escapes. The highest transformation efficiency (22%) yielding the largest quantity of transgenic rice plants (54) was obtained when hygromycin B at 50 mg L^{-1} was used as a selection agent, indicating that hygromycin did not inhibit plant regeneration of stably transformed calli. In addition, 96 % of hygromycin resistant plants were resistant to glufosinate, indicating that hygromycin used as an indirect selection agent for glufosinate resistance still allowed a few escapes. Similarly, a low concentration (25 mg L^{-1}) of hygromycin resulted in low transformation efficiency (2%) and a low percentage (14 %) of glufosinate resistant plants.

In our experiments, hygromycin B at 50 mg L^{-1} was an efficient selection agent to recover glufosinate resistant plants. This was consistent with previous reports in developing transgenic rice for resistance to rice pink stem borer, stripped stem borer (*Chilo suppressalis*), yellow stem borer (Cheng et al.,1998; Duan et al., 1996; Wu et al., 1997), the sheath blight pathogen, *Rhizoctonia solani* (Lin et al., 1995), and nematode (M*eloidogyne incognita*) (Vain et al., 1998), in that the *hph* gene was used exclusively as a selection marker. In contrast, there have been few successful reports of using bialaphos as a selectable marker gene in rice transformation (Rathore et al. 1993; Toki et al., 1992). Selection with bialaphos using the *bar* gene as a selection marker was not successful in transformation of the Australian rice cultivar Jarrah (Abedinia et al., 1997), though bialaphos was successfully used as a selection agent in recovery of other cereal crops including maize (*Zea mays*) (Gordon-Kamm et al., 1990), wheat (*Triticum aestivum*) (Weeks et al.,1993), barley (Wan and Lemaux, 1994), and sugarcane (*Saccharum*

officinarum) (Gallo-Meagher and Irvine, 1996). The previously reported efficient, routine, or consistent systems/protocols for production of transgenic rice plants for the Australian rice cultivar Jarrah (Abedinia et al., 1997), diverse *indica* or tropical *japonica* cultivar (Ghosh Biswas et al.,1998; Sivamani et al., 1996; Zhang et al., 1996;) or *japonica* cultivar Taipei 309 (Chen et al., 1998) were all exclusively dependent on selection for hygromycin resistance mediated by the *hph* maker gene. Numerous transgenic rice plants have been produced repeatedly in our experiments by employing bialaphos selection, demonstrating that this compound is also an effective and reliable selection agent for transformation of elite U.S. rice lines. This result may be important for the commercial production of transgenic rice where presence of the *hph* gene is undesirable.

2.3.4 Production of fertile transgenic rice plants with glufosinate resistance

Bombardment-based transformation of embryogenic calli (2-3 mm in size) induced from mature seed embryos in CI medium, in conjunction with hygromycin or bialaphos selection, was productive and reproducible. A summary of the transformation experiments for 6 elite rice lines is presented in Table 2.3. Cocodrie and medium-grain elite line LA9502065 were the first two lines used extensively in establishing an efficient transformation system for production of transgenic glufosinate resistant lines. The transformation system was also shown to be reproducible in the remaining four elite lines, LA9502002, 96URN082, 96URN085, and 96URN131. A total of 5201 embryogenic calli were bombarded with plasmid vectors containing the *bar* (pAHC25) or *pat* (pPAT63 and pB2/35SAck) gene. Stable transformants were recovered by using either bialaphos or hygromycin as a selective agent. The transformation frequency, as defined in terms of

Table 2.3. Number of calli bombarded, hygromycin- or bialaphos-resistant calli, and glufosinate-resistant plants for six transgenic elite U.S. rice breeding lines.

Rice line	Pedigree	Exp. no.	Vector	No. of calli bombarded (A)	No. of Hyg-R or BL-R calli (B)	No. of calli produced Hyg-R or BL-R plants (C)	No. of glufosinate-resistant plants (D)	Transformation frequency (B/A)	Transformation efficiency (D/A)
Cocodrie	Cypress//82CAY21/Tebonnet	1	pAHC25+pTRA151	210	3	2	14	0.014	0.067
		2	pAHC25+pTRA151	462	21	16	43	0.045	0.093
		3	pAHC25+pTRA151	384	20	11	89	0.052	0.232
		4	pPAT63	180	28	21	133	0.156	0.739
		5	pPAT63	150	15	14	155	0.100	1.033
		6	pB2/35SAcK	160	12	9	28	0.075	0.175
		7	pB2/35SAcK	150	8	4	16	0.053	0.107
LA9502065	Bengal/Rico1	1	pAHC25+pTRA151	391	30	19	210	0.077	0.537
		2	pAHC25+pTRA151	340	43	9	17	0.126	0.050
		3	pAHC25+pTRA151	684	23	21	165	0.034	0.241
		4	pPAT63	507	37	18	25	0.073	0.049
		5	pPAT63	250	12	12	54	0.048	0.216
		6	pPAT63	500	16	15	78	0.032	0.156
LA9502002	Maybelle//Lemont/2001-5	1	pPAT63	96	3	3	5	0.031	0.052
		2	pPAT63	108	9	7	43	0.083	0.398
96URN082	Cypress//82CAY21/Tebonnet	1	pPAT63	120	28	24	80	0.233	0.667
		2	pPAT63	115	13	8	26	0.113	0.226
96URN085	Cypress//L201/7402003	1	pPAT63	90	20	19	45	0.222	0.500
		2	pPAT63	124	15	5	8	0.121	0.065
96URN131	Mercury/Rico 1	1	pPAT63	90	19	15	25	0.211	0.278
		2	pPAT63	90	14	6	9	0.156	0.100
Total				5201	389	258	1268	0.075	0.244

Fig. 2.5. Transgenic R0 rice plants of Cocodrie grown to maturity in the greenhouse.

number of bialaphos- or hygromycin-resistant calli in 100 bombarded calli, ranged from 1% (LA9502065) to 23% (96URN082). A total of 389 bialaphos- or hygromycin-resistant calli were obtained, giving an average transformation frequency of 7% (389/5201). These bialaphos- or hygromycin-resistant calli were highly embryogenic. After being transferred onto the regeneration media, 66 % (256/389) of these stable transformants readily differentiated into plantlets in the presence of bialaphos (4 mg L^{-1}) or hygromycin (50 mg L^{-1}). A total of 1268 R0 plants resistant to the herbicide glufosinate (2000 mg L^{-1}) in the greenhouse were obtained. A total of 62% (785/1268) of transgenic rice plants grew to maturity and set seeds normally (Fig 2.5). The efficiency of stable transformation, as defined (Sivamani et al., 1996) in terms of the number of hygromycin- or bialaphos-

resistant R0 plants regenerated from 100 bombardment explants, ranged from 5% (LA952065) to 100% (Cocodrie). The average transformation efficiency across all 6 lines was 24% (1268 plants/5201 calli), which was higher than those in previous reports (3 % for TN1, Sivamani et al., 1996; 22% for Taipei 309, Chen et al., 1998).

2.3.5 Southern blot analysis of the *pat* gene in R0 and R1 glufosinate resistant plants

Four R0 plants representing 4 independent transgenic lines and 6 R1 plants derived from one fertile transgenic line were analyzed for stable incorporation of the *pat* gene in a Southern blot analysis (Fig.2.6). Both undigested (lanes15-19) and digested (lanes 4-15) rice genomic DNA from the transgenic rice lines and untransformed cultivar Cocodrie were included in the blot. When hybridized with the *pat* gene probe, the untransformed plant did not show any hybridization band in the *Eco*R I digested (lane 4) or undigested (lane 15) genomic DNA, whereas the undigested (lanes 16-19) genomic DNAs of 4 independent glufosinate resistant plants appeared as a smear in the regions of high molecular weight DNA, providing evidence that the *pat* gene was stably integrated into the genomic DNA. As expected, the *Eco*R I digestion of pPAT63 (lanes 1-3) and genomic DNAs (lanes 11-14) of pPAT63 transgenic rice plants generated a 1.4 kb fragment that contained the intact *pat* gene expression unit. However, the banding pattern differed among the four R0 plants, which confirmed that these R0 plants represented 4 independent transgenic lines. Compared to Line 1016 (lane 11) and Line 1001 (lane12), the pattern of bands in Line1017 (lane 13) and Line 1026 (lane 14) indicated complex integration events of the *pat* gene. The presence of multiple weaker bands of higher

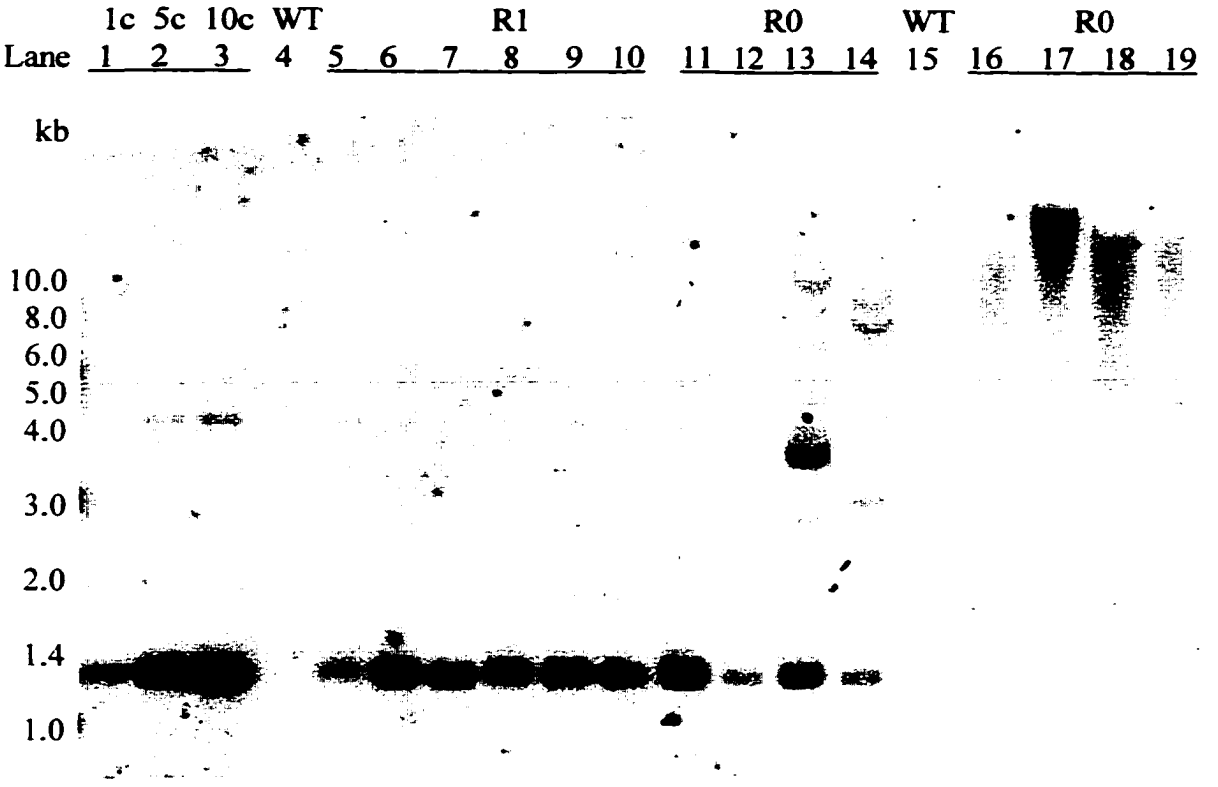

Fig. 2.6. Southern blot analysis of the *pat* gene in R0 and R1 transgenic plants of Cocodrie. Lanes 1-3: plasmid pPAT63 digested with *Eco*R I, equivalent to 1, 5 and 10 copies of the *pat* gene per rice haploid genome. Lanes 4-14, genomic DNAs digested with *Eco*R I; Lane 4: a wild-type (untransformed) plant; Lanes 5-10: 6 R1 progeny plants, 16-1, 16-2, 16-3, 16-4, 16-5 and 16-6 derived from the transgenic line 1016 (R0, Lane11); Lanes 11-14: 4 independent R0 transgenic lines, 1016, 1001, 1017, and 1026; Lane 15: undigested genomic DNA of the wild-type plant; Lanes 16-19, undigested genomic DNAs from 4 independent R0 transgenic lines corresponding to Lanes 11-14. DNAs were electrophoresed in 1.0% agarose gel, transferred onto Hybond-NX membranes (Amersham), and hybridized with P^{32}-labeled 0.45 kb *Sma* I fragment from pPAT63.

molecular weight may represent rearranged copies of the *pat* gene expression unit or partial digestion of the rice genomic DNA with *Eco*R I. The complex insertion of the transgene may be responsible for the phenotypical abnormalities in the corresponding transgenic lines. For example, both Line 1017 and Line 1026 were sterile though highly resistant to glufosinate in the greenhouse and field, whereas Line 1016 and L1001 were fertile and phenotypically normal. Compared with untransformed Cocodrie, Line 1017 was dwarf with excessive tillering while Line 1026 was tall and poor in tillering.

DNA hybridization profiles (lanes 5-10) of 6 R1 progeny, 16-1, 16-2, 16-3,16-4, 16-5, 16-6, and their corresponding parent (R0) plant Line 1016 (lane 11) were found to be identical (Fig.2.6), providing molecular evidence that the *pat* gene was transmitted intact to the R1 progeny.

2.3.6 Southern blot hybridization analysis for presence of the *hph* selection marker gene in R0 and R1 glufosinate resistant plants

Because the selectable marker gene *hph* is linked to the *pat* gene in plasmid pPAT63, re-hybridization of genomic DNA of the above sampled transgenic lines with *hph* probe was carried out to further analyze the transgenic events. Digestion of pPAT63 and genomic DNA of pPAT63-transformed rice plant and their progeny with *Eco*R I should release a 4.4-kb fragment containing the intact 1.7-kb *hph* gene cassette. As expected, hybridization was observed in a 4.4-kb band of *Eco*R I digested genomic DNAs and in the region of higher molecular weight of undigested genomic DNA (Fig. 2.7). These results demonstrated that the *hph* gene was stably integrated into the rice genome.

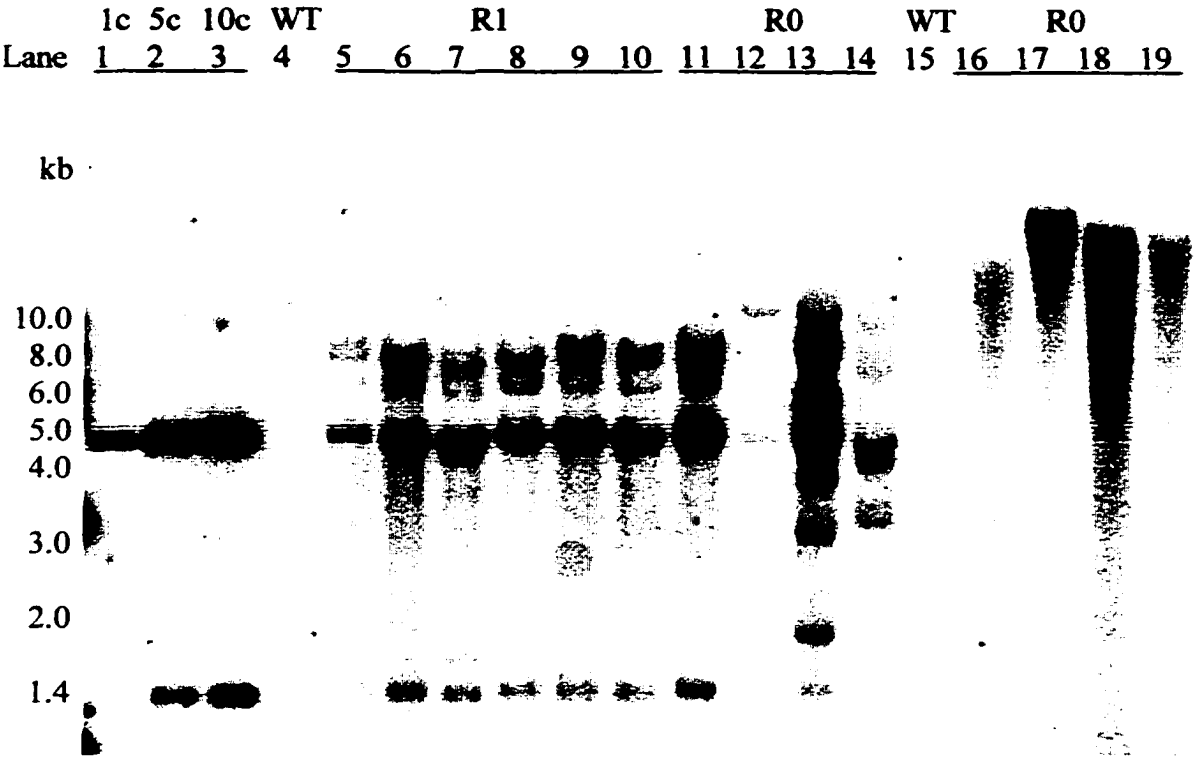

Fig. 2.7. Southern blot analysis of the *hph* gene in R0 and R1 transgenic plants of Cocodrie. Lanes 1-3: plasmid pPAT63 digested with *Eco*R I, equivalent to 1, 5 and 10 copies of the *pat* gene per rice haploid genome. Lanes 4-14, genomic DNAs digested with *Eco*R I; Lane 4: a wild-type (untransformed) plant; Lanes 5-10: 6 R1 progeny plants, 16-1, 16-2, 16-3, 16-4, 16-5 and 16-6 derived from the transgenic line 1016 (R0, Lane11); Lanes 11-14: 4 independent R0 transgenic lines, 1016, 1001, 1017, and 1026; Lane 15: undigested genomic DNA of the wild-type plant; Lanes 16-19, undigested genomic DNAs from 4 independent R0 transgenic lines corresponding to Lanes 11-14. DNAs were electrophoresed in 1.0% agarose gel, transferred onto Hybond-NX membranes (Amersham), previously hybridized with a *pat* probe. After stripping, the blot was re-hybridized with P^{32}-labeled 1.1 kb *Hind* III fragment of pTRA151.

We detected variation in copy number of the intact or rearranged *pat* gene among four independent lines (Fig.2.6 and Fig. 2.7), but their resistance to glufosinate was similar. This was consistent with previous reports (Nagy et al., 1985; Spencer et al.,1990) that found no apparent correlation between the copy number of intact or rearranged *bar* genes and the level of expression in transgenic plants.

2.3.7 Germination test of mature seeds of R1 generation

Transmission of the *pat* or *bar* gene was first demonstrated by germination of R1 seeds of one transgenic line G8T developed from LA9502065 in the presence of 4 mg L^{-1} bialaphos. A total of 22 of 30 seeds tested germinated in the rooting medium RT-B4 (containing 4 mg L^{-1} bialaphos) as vigorously as in the absence of bialaphos, but no seeds of untransformed LA9502065 germinated in the same medium. A 3:1 ratio of glufosinate resistance (22): susceptibility (8) observed in germination test of R1 seeds and the Southern blot hybridization analysis confirmed that the *pat* or *bar* gene was integrated into the rice genome and transmitted intact to the progeny.

2.3.8 Segregation and field evaluation of glufosinate resistance in R1 and R2 progeny

Segregation of glufosinate resistance was further investigated in the R1 and R2 progeny. Selfed progeny (R1) of 20 transgenic lines of Cocodrie (Tables 2.4, 2.5) and 10 transgenic lines of LA9502065 (Table 2.6) were examined for glufosinate resistance in field plots at the Rice Research Station, Crowley, LA in 1998. Plants were sprayed at a rate of 1.12 kg ha^{-1} (1.0 lb ai/A) at the 3 to 4 leaf stage. Resistant and susceptible plants were scored 7 DAT (Fig. 2.8) and susceptible plants died within 2 wk after treatment.

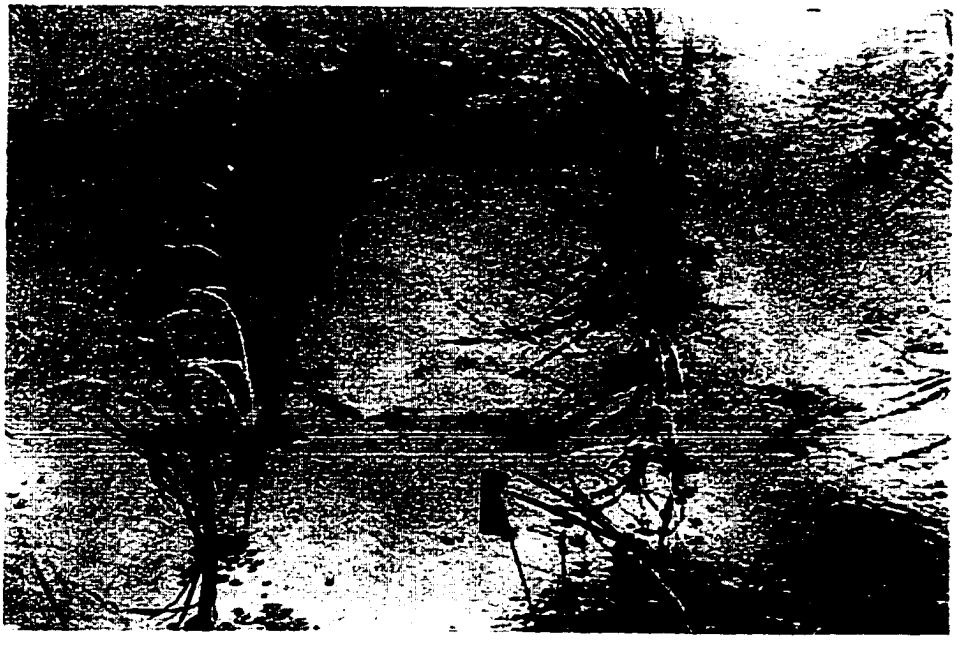

Fig. 2.8. Segregation of glufosinate resistance in R1 progeny from selfed R0 seeds at the Rice Research Station, Crowley, LA, 1998.

For transgenic Cocodrie derived material, glufosinate resistance segregated in all 17 R1 progeny with 3:1, 9:7, or 15:1 χ^2 ratios (Tables 2.4, 2.5). In the R2 generation, 85% (53/62) of the lines segregated for one or two dominant genes as in the R1, but not all R2 progeny segregated for resistance like their parental R1 lines. For example, R1 line 617-1 segregated 3:1 for glufosinate resistance, but 9:7 and 15:1 ratios were also observed the R2 generation (Table 2.5). Sixty seven percent (6/9) of all abnormal R2 segregation was detected in lines derived from two R1 parents, C02 and 703-4. Similar to Cocodrie R1 progeny, glufosinate resistance in R1 lines of LA9502065 was controlled by one or two dominant genes (Table 2.6). However, only 37% (14/38) of the R2 progeny exhibited

Table 2.4. Segregation of glufosinate resistance in R1 and R2 transgenic Cocodrie derived lines, A01-G06, 1998, Rice Research Station, Crowley, LA; 1999, Lajas, Puerto Rico.

Generation	Line	No. of plants sprayed	No. of plants resistant	No. of plants susceptible	X^2 ratio†	X^2 value‡
R1	A01	15	12	3	3:1	0.200
R2	A01-1	40	22	18	9:7	0.025
	A01-2	34	19	5	3:1	3.098
	A01-3	30	18	12	9:7	0.171
	A01-3-GH	30	23	7	3:1	0.044
R1	A02	15	10	5	3:1	0.556
R2	A02-1-1-GH	32	32	0	stable	-
	A02-1-1	26	19	7	3:1	0.051
	A02-1-2	22	14	8	3:1	1.515
R1	C01	19	10	9	9:7	0.101
R2	C01-1	28	22	6	3:1	0.190
R1	C02	30	21	9	3:1	0.400
R2	C02-3	37	36	1	-	-
	C02-4	100	84	16	-	-
	C02-5	30	9	21	-	-
R1	D01	25	13	12	9:7	0.183
R2	D01-1	24	18	6	3:1	0.000
R1	D05	19	13	6	3:1	3.813
R2	D05-1	37	19	18	9:7	0.361
R1	G01	44	31	12	3:1	0.212
R2	G01-1	36	32	4	3:1	3.704
	G01-3	24	16	8	3:1	0.889
R1	G03	41	35	16	3:1	3.813
R2	G03-1-1	71	61	10	-	-
R1	G05	22	18	4	3:1	0.545
R2	G05-1	41	34	7	3:1	1.374
	G05-2	40	38	2	15:1	0.107
R1	G06	27	17	10	9:7	0.494
R2	G06-4	10	8	2	3:1	0.133

† Best Chi square ratio of those tested; A '-' indicates no ratio fit.

‡ df=1, X^2=3.841 at the 0.05 probability level.

Table 2.5. Segregation of glufosinate resistance in R1 and R2 transgenic Cocodrie derived lines 617-1, 617-2, 617-6, 617-8, 618-1, 703-3, and 703-4, 1998, Rice Research Station, Crowley, LA; 1999, Lajas, Puerto Rico.

Generation	Line	No. of plants sprayed	No. of plants resistant	No. of plants susceptible	X^2 ratio†	X^2 value‡
R1	617-1	14	11	3	3:1	0.095
R2	617-1-1	9	8	1	15:1	0.363
	617-1-2	18	11	7	9:7	0.173
	617-1-5	23	18	5	3:1	0.130
	617-1-6	36	16	20	9:7	2.039
	617-1-7	52	32	20	9:7	0.591
	617-1-8	21	13	8	9:7	0.273
	617-1-10	39	17	22	9:7	2.540
	617-1-11	38	34	4	15:1	1.186
R1	617-2	13	9	4	9:7	0.890
R2	627-2-1	30	25	5	3:1	1.111
	617-2-4	33	24	9	3:1	0.091
	617-2-5	31	22	9	3:1	0.269
	617-2-9	37	27	10	3:1	0.081
	617-2-10	37	19	18	9:7	0.361
	617-2-11	25	20	5	3:1	0.333
R1	617-6	18	13	5	3:1	0.074
R2	617-6-2	22	16	6	3:1	0.061
	617-6-6	39	23	16	9:7	0.118
	617-6-7	38	26	12	3:1	0.877
	617-6-10	108	70	38	9:7	3.219
	617-6-11	71	58	13	3:1	1.695
	617-6-12	44	27	17	9:7	0.468
	617-6-13	43	34	9	3:1	0.380
	617-6-14	26	16	10	9:7	0.295
	617-6-16	52	42	10	3:1	0.923
R1	617-8	35	25	10	3:1	0.238
R2	617-8-1	25	18	7	3:1	0.120
	617-8-2	29	23	6	3:1	0.287
	617-8-3	51	37	14	3:1	0.163
	617-8-4	25	17	8	3:1	0.653
	617-8-5	24	20	4	3:1	0.889
	617-8-6	38	24	14	9:7	0.737
	617-8-10	24	18	6	3:1	0.000
	617-8-13	19	10	9	9:7	0.101
	617-8-16	32	20	12	9:7	0.508
	617-8-22	33	29	4	15:1	1.941
	617-8-23	28	26	2	15:1	0.038
	617-8-25	36	22	14	9:7	0.346
	617-8-27	35	32	3	15:1	0.322
	617-8-28	27	19	8	3:1	0.309
	617-8-29	19	13	6	3:1	0.439
R1	618-1	14	13	1	15:1	0.019
R2	618-1-8	10	6	4	-	-
R1	703-3	31	29	3	15:1	0.583
R2	703-3-3	20	15	5	3:1	0.000
	703-3-6-GH	7	7	0	stable	-
R1	703-4	58	52	6	15:1	1.660
R2	703-4-2	2	1	1	-	-
	703-4-3-GH	16	16	0	stable	-
	703-4-18	7	2	5	-	-

† Best Chi square ratio of those tested; A '-' indicates no ratio fit.

‡ df=1, x^2=3.841 at the 0.05 probability level.

Table 2.6. Segregation of glufosinate resistance in R1 and R2 transgenic LA9502065 derived lines I02, K07, L01, L02, L03, L04, L05, L08, and L11,1998, Rice Research Station, Crowley, LA; 1999, Lajas, Puerto Rico.

Generation	Line	No. of plants sprayed	No. of plants resistant	No. of plants susceptible	X^2 ratio†	X^2 value‡
R1	I02	10	6	4	9:7	0.057
R2	I02-2	48	38	10	3:1	0.444
R1	K07	16	9	7	3:1	3.000
R2	K07-2	35	27	8	3:1	0.086
R1	L01	26	16	10	9:7	0.295
R2	L01-N-3	44	44	0	stable	-
	L01-N-4	48	4	44	-	-
	L01-N-6	88	13	75	-	-
	L01-N-7	56	20	36	-	-
	L01-N-8	64	5	59	-	-
	L01-N-9	64	14	50	-	-
	L01-N-10	64	11	53	-	-
	L01-N-11	136	18	118	-	-
	L01-N-12	80	12	68	-	-
R1	L02	12	10	2	3:1	0.444
R2	L02-D-4	64	10	54	-	-
	L02-D-6	64	3	61	-	-
	L02-D-7	72	39	33	9:7	0.127
	L02-D-8	88	31	57	-	-
	L02-D-10	112	21	91	-	-
	L02-D-12	48	6	42	-	-
	L02-D-13	100	64	36	9:7	2.441
	L02-D-15	110	68	42	9:7	1.386
	L02-D-16	130	85	45	-	-
	L02-D-17	130	88	42	3:1	3.703
	L02-D-18	150	45	105	-	-
	L02-D-20	140	104	36	3:1	0.038
R1	L03	25	21	4	3:1	1.080
R2	L03-N-1	50	7	43	-	-
	L03-N-4	90	64	26	3:1	0.726
	L03-N-5	140	112	28	3:1	1.867
	L03-N-6	80	28	52	-	-
	L03-N-7	99	99	0	stable	-
	L03-6	63	28	35	-	-
	L03-5	51	37	14	3:1	0.163
R1	L04	12	11	1	15:1	0.089
R2	L04-D-1	32	9	23	-	-
	L04-D-2	40	5	35	-	-
	L04-D-3	56	18	38	-	-
	L04-D-4	80	31	49	-	-
R1	L05	16	9	7	9:7	0.000
R2	L05-D-1	24	12	12	9:7	0.381
	L05-D-2	40	20	20	9:7	0.635
	L05-D-3	69	55	14	3:1	0.816
R1	L08	28	20	8	3:1	0.190
R1	L11	15	11	4	3:1	0.022

† Best Chi square ratio of those tested; A '-' indicates no ratio fit.

‡ df=1, X^2=3.841 at the 0.05 probability level.

known segregation patterns. Over one half of the abnormal R2 segregation ratios (13/24, 54%) could be traced to R1 lines L01 and L04. Reasons for the observed differences in segregation for glufosinate resistance in Cocodrie vs. LA9502065 are unknown, but differences in genetic background of the two lines may play a role.

In 5 (17%) out of 30 independent transgenic events, i.e., B01, F01, H01, J01 and M01, all R1 plants were susceptible to glufosinate (data not shown), despite the fact that their parental R0 plants were regenerated in the presence of bialaphos and resistant to glufosinate at 1000 mg L^{-1} in the greenhouse. These results indicated escapes or gene silencing in these 5 transgenic lines. Although no molecular evidence was produced to elucidate gene silencing in this experiment, the aberrant or inconsistent segregation patterns for glufosinate resistance from R1 to R2 progeny derived from the same primary transformants was most likely the result of gene silencing. Kumpatla et al. (1997) studied bar expression in rice and demonstrated that a transgene locus containing multiple rearranged copies was functional in primary transformants but was readily methylated and frequently silenced in subsequent generations.

2.3.9 Recovery of homozygous glufosinate resistant lines in R2 progeny

R2 progeny plants of 4 Cocodrie transgenic rice lines, A02-1, C02-3 (Table 2.4), 703-3-6, and 703-4-3 (Table 2.5), and 2 LA9502065 transgenic rice lines, L01-N-3 and L03-N-7, exhibited homogenous resistance to glufosinate in the greenhouse or field test. These 6 R2 populations were apparently derived from 6 homozygous R1 plants. They

grew to maturity and set seeds normally (Fig. 2.9) under field conditions. Stability of glufosinate resistance in these materials has been confirmed in the R3 and R4 generations (data not shown).

Fig. 2.9. Stable, elite transgenic rice lines (R2) were recovered and grown to maturity at the Rice Research Station, Crowley, LA, 1998.

2.3.10 Abnormal transgenic lines

Particle bombardment of rice mature seed-derived embryogenic calli generated glufosinate resistance in primary transgenic plants and their progeny with both normal and aberrant phenotypes, such as dwarfism, over-tillering, sterile, straight-headed, and purple-lemma of seeds. Several R1 progeny plants resistant to glufosinate in the field set purple seeds whereas their parental R0 plants set seeds with normal color. Some R1 plants set seeds of both normal color and purple or tan while some plants produced all purple seeds. The segregation of purple to normal varied from panicle to panicle within a single R1 plant and between individual R1 progeny plants from the same R0 parental

plant. The ratio of purple seeds in a single panicle varied from 24% to 78%. It was observed that the majority (~90%) of purple seeds were sterile, more susceptible to fugal disease, exhibiting a possible linkage of fecundity and coloration of seeds. However, stable lines with glufosinate resistance and purple-seed characteristic were recovered in R2-R3 progeny, indicating that purple-seed character is heritable and may be used as a unique phenotypic marker for glufosinate resistance. The putative mutation may be caused by insertion of the *pat* or *bar* gene into other genes related to fecundity and pigmentation of seeds.

CHAPTER 3. HIGH EFFICIENCY TRANSFORMATION OF ELITE U.S. RICE LINES MEDIATED BY *AGROBACTERIUM* AND FIELD EVALUATION OF TRANSGENIC LINES

3.1 Introduction

Agrobacterium-mediated genetic transformation of plants is an attractive alternative to direct DNA delivery methods. The advantages of *Agrobacterium*-mediated gene transfer over other methods include high efficiency of transformation, transfer of pieces of DNA with defined ends at low copy number, transfer of relatively large segments of DNA, and the absence of a requirement for protoplast culture technique or any special equipment (Hiei et al., 1997; Shimamoto, 1995). Application of an *Agrobacterium*-mediated transformation system, however, had been until recently limited to dicotyledonous plants. Monocotyledonous plants, in particular cereal plants including rice, are not the natural hosts of *Agrobacterium tumefaciens*. Advances in tissue culture methodology and development of more potent T-DNA vectors, such as a 'super-binary' vectors, have rendered some cereal species amenable to *Agrobacterium*. Transgenic plants of rice (*Oryza sativa*) (Hiei et al., 1994), maize (*Zea mays*) (Ishida et al., 1996), wheat (*Triticum aestivum*) (Cheng et al., 1997), and barley (*Hordeum vulgare*) (Tingay et al., 1997) have been successively obtained via *Agrobacterium*-mediated transformation. Transgenic rice plants have been produced from *indica, japonica* (Aldemita and Hodges, 1996; Rashid et al., 1996), and *tropical japonica* (Dong et al., 1996) subspecies. Using *Agrobacterium*-mediated transformation, certain important agronomic traits, such as resistance to striped stem borer (*Chilo suppressalis*) and yellow stem borer (*Scirpophaga*

incertulas) (Cheng et al., 1998), C4-photosynthesis (Ku et al., 1999), and unsaturation of fatty acids and chilling tolerance of photosynthesis (Yokoi et al., 1998) have been introduced into rice. However, *Agrobacterium*-mediated transformation of rice is not yet routine and the explants are limited to immature embryo- or scutellum-derived calli (See review by Hiei et al., 1997; Yokoi et al., 1998). The potential for *Agrobacterium* transformation using anther-derived cultures has not been adequately studied (Balconi et al., 1998). Currently, there is no report of field performance of *Agrobacterium* transformed rice plants which is essential to evaluate the feasibility of the protocol for rice genetic engineering. The need to work directly with elite breeding lines rather than the model genotypes, regardless of their amenability to tissue culture, is also warranted.

The objectives of this study are to establish a routine, efficient *Agrobacterium*-mediated transformation method for U.S. elite rice lines as an alternative to particle bombardment and to examine the competence of anther-derived calli for *Agrobacterium* transformation. A large number of transgenic calli and plants have been repeatedly produced from both anther- and scutellum-derived calli, which substantiated that *Agrobacterium*-mediated transformation of rice via scutellum-derived calli (Hiei et al., 1994) is a feasible alternative to particle bombardment for production of transgenic U.S. rice lines. Furthermore, our research demonstrated for the first time the competence of anther cultures as explants for *Agrobacterium*-mediated transformation, though the problem of albinism in transgenic rice plants should be addressed in the future studies. In addition, a majority of R2 progeny of 3 transgenic lines derived form LA9502065 transformed by *Agrobacterium tumefaciens* strain LBA4404 (pTOK233) exhibited

normal agronomic traits and less morphological variation than those produced from bombardment experiments in a preliminary field trail, indicating *Agrobacterium* could be utilized for introduction of useful genes into rice.

3.2 Materials and Methods

3.2.1 Plant materials and *in vitro* callus production

3.2.1.1 Rice genotypes and culture media

Two elite U.S. breeding lines LA9502065 and LA9502008 (Cocodrie), six F_1 lines, namely, ACF112, ACF157, ACF179, ACF182, ACF186, ACF222, and one F2 line, ACF126F_2 were used in the transformation studies. Media used for tissue culture and transformation are listed in Table 3.1.

3.2.1.2 Calli and suspension cultures derived from scutella

Mature seeds were dehusked and surface-sterilized in 50% (v/v) commercial bleach (containing 6.25 % sodium hypochlorite) for 45 min followed by 3 rinses in sterile distilled water. Seeds were aseptically plated on CI medium (Table 3.1) for callus induction. After 2 wks in the dark at 26 °C, primary calli initiated on the scutellar surface of mature seed embryos were selected and subcultured for another 2 wks on fresh CI medium. Unless specifically stated, the proliferated calli were then divided into small pieces (approximately 2-3 mm in diameter) and subcultured on fresh CI medium for another 3 days. Only actively growing, one-month-old calli were used for transformation experiments. To investigate effect of cell age and cell type on transformation efficiency, 2-3 mm pieces of embryogenic calli were subcultured every 3 wks on fresh CI medium

Table 3.1. Composition of media for tissue culture and transformation in this study.

Medium	Composition
CI	CC salts and vitamins (Potrykus et al. 1979), 20 g L^{-1} sucrose, 18.2 g L^{-1} mannitol, 18.2 g L^{-1} sorbitol, 2 mg L^{-1} 2,4-D, 7 g L^{-1} agarose (Type I-A, Sigma), pH 5.8
CHU	Chu salts and vitamins (Chu et al., 1997), 20 g L^{-1} sucrose, 20 g L^{-1} sorbitol, 20 g L^{-1} maltose, 1 mg L^{-1} 2,4-D, , 0.1 mg L^{-1} zeatin, 2 g L^{-1} phytagel (Sigma), pH 5.8
R203	R2 salts and vitamins (Ohira et al., 1973), 20 g L^{-1} sucrose, 10 g L^{-1} maltose, 560 mg L^{-1} proline, 1 mg L^{-1} 2,4-D, pH5.8
AB	AB salts (Chilton et al., 1974), 5 g L^{-1} glucose, 15 g L^{-1} Bacto-agar (Difco Laboratories), pH 7.2
LB	LB Broth (Miller, 1972), 15 g L^{-1} Bacto-agar, pH 7.2
AAM	AA salts and amino acids (Toriyama and Hinata, 1985), MS vitamins (Murashige and Skoog, 1962), 500 mg L^{-1} casein enzymatic hydrolysate, 68.5 g L^{-1} sucrose, 36 g L^{-1} glucose, 100 µM Acetosyringone (3', 5'-dimethoxy-4'-hydroxy acetophenone, Aldrich Chem, Co. USA), pH5.2
CI-AS	CI with 100 µM Acetosyringone, pH 5.2
CI-CH50	CI with 50 mg L^{-1} hygromycin B (Boehringer Mannheim), 250 mg/l cefotaxime (Claforan, Hoechst-Russel, Germany), pH 5.8
PR1-H50	CC salts and vitamin (Potrykus et al. 1979), 1.0 g L^{-1} casein hydrolysate, 20 g L^{-1} sucrose, 36.4 g L^{-1} sorbitol, 1 mg L^{-1} IAA, 0.05 mg L^{-1} zeatin ribosome, 7 g L^{-1} agarose, 50 mg L^{-1} hygromycin B, pH 5.8
PR2-H50	MS salts and vitamins (Murashige and Skoog, 1962), 20 g L^{-1} sucrose, 10 g L^{-1} maltose, 2 mg L^{-1} kinetin, 0.5 mg L^{-1} NAA, 7 g L^{-1} agarose, 50 mg L^{-1} hygromycin B, pH 5.8
RT-H50	MS salts and vitamins (Murashige and Skoog, 1962), 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel (Sigma), 50 mg L^{-1} hygromycin B, pH 5.8

or suspended in a modified R2 liquid medium (Table 3.1) and cultured on a rotary shaker (80 rpm) at 26 °C. The suspension cells were subcultured every 7 days with fresh R2 medium. Calli or suspension cultures in the logarithmic phase of growth (3-4 days after subculture for 2 to 5 months) were evaluated in transformation experiments.

3.2.1.3 Calli and suspension cultures derived from anthers

Panicles from rice plants grown at the LSU Rice Research Station at Crowley, LA were picked at the booting stage when microspores were at the mid-to-late uninucleate stage (the flag leaf node has emerged approximately 0.5 to one inch). Panicles were harvested generally from 9 to11 AM. All except the top two leaves were removed from harvested panicles that were wrapped in moistened paper towels, placed in plastic bags, and subjected to cold pre-treatment at 4 °C for 7-10 days. The panicles were then surface-sterilized in 50% (v/v) commercial bleach (containing 6.25 % sodium hypochlorite) for 15 min. After removal of the remaining leaves around panicles, spikelets were dissected under sterile conditions and anthers were inoculated on CHU medium (Table 3.1) for callus induction. After 4-6 weeks, calli of 1-1.5 mm in diameter were subcultured on fresh CHU medium every 3 wks or suspended and subcultured in R2 liquid medium on a rotary shaker (80 rpm). Calli or suspension cultures in the logarithmic phase of growth (3-4 days after subculture for 2 to 5 months) were tested in transformation experiments. Primary calli for six F_1 lines, namely, ACF112, ACF157, ACF179, ACF182, ACF186, ACF222, and one F_2 line ACF126F_2 were supplied by Dr. Qiren Chu of the Rice Biotechnology Laboratory, LSU Rice Research Station, Crowley, LA.

3.2.2 *Agrobacterium* strain and plasmid

Agrobacterium tumefaciens strain LBA4404 (Hoekema et al., 1983) containing a 'super-binary' vector pTOK233 (Hiei et al.,1994) was used in this experiment. This strain has been successfully used in transformation of rice (Hiei et al., 1994) and maize (Ishida et al., 1995). The T-DNA of pTOK233 (Fig.3.1) contains a hygromycin-resistance gene (*hph*), a kanamycin-resistance gene (*npt*), a GUS gene, which has an intron in the N-terminal region of the coding sequence and is fused to the CaMV35S promoter (Odell et al., 1985). The *intron-gus* gene expresses GUS activity in plant cells, but not in cells of *A. tumefaciens* (Ohta et al., 1990).

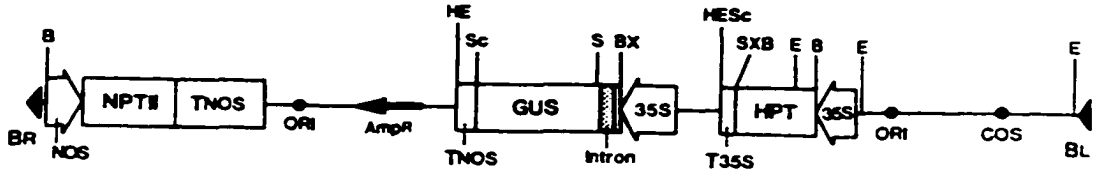

Fig. 3.1. T-DNA region of pTOK233. Abbreviations: BR, right border; BL, left border; NPT II, neomycin phosphotransferase; GUS, β-glucuronidase; NOS, nopaline synthase promoter; TNOS, 3' signal of nopaline synthase; T35S, 3' signal of 35S RNA; ORI, origin of replication of replication of ColE1; AmpR, ampicillin-resistance gene active in *E. coli*; B, *Bam*HI; E, *EcoR*I; H, *Hind*III; S, *Sal*I; Sc, *Sac*I; X, *Xba*I.

3.2.3 *Agrobacterium* virulence induction and co-cultivation with plant tissues

Unless otherwise stated, *Agrobacterium tumefaciens* LBA4404(pTOK233) was grown for 3 days at 28 °C on AB medium (Chilton et al., 1974) supplemented with 50 mg

L^{-1} hygromycin B (Boehringer Mannheim) and 15 g L^{-1} agar, pH 7.2. The bacteria were collected and suspended at 1.5-2.0 x 10^9 cells ml^{-1} in pre-induction AAM medium (Table 3.1). Calli or suspension cultures derived from scutella or anthers were immersed in the bacterial suspension for approximately 5 min, transferred without rinsing to co-cultivation medium CI-AS (Table 3.1), and incubated in the dark at 26 °C for 3 days. Alternatively, calli or suspension cultures were first plated onto the co-cultivation medium, and bacterial suspensions as inoculum were thereafter applied to targets, 10 µl of inoculum per calli or approximately 1 ml per 1 gram of cell suspensions.

3.2.4 Selection and regeneration of transformed cells

After co-cultivation, calli or suspension cultures were collected, rinsed thoroughly with CI liquid medium containing 250 mg/l cefotaxime (Claforan, Hoechst-Russel, Germany) and placed on selection medium CI-CH50. Cultures were incubated at 26 °C in darkness for up to 3 wks and transferred to fresh CI-CH50 medium for another 2 to 3 wks. At that point, actively growing, hygromycin-resistant calli were recovered, whereas all untransformed calli were completely inhibited and turned necrotic. Transformation frequency was defined as the percent of hygromycin-resistant calli. To obtain transgenic plants, embryogenic calli were selected and placed on regeneration medium PR1-H50 or PR2-H50 (Table 3.1.) and incubated at 26 °C under a 16 h photoperiod. In approximately 3 wks, regenerated shoots were obtained and transferred to rooting medium RT-H50 for 2 to 3 wks and finally transferred to soil in pots and grown to maturity in a greenhouse.

3.2.5 Confirmation of stable transformants with GUS assay

To confirm that hygromycin-resistant calli on the selection medium and plantlets regenerated from them are stable transformants rather then mutants, expression of GUS in rice calli, leaves, and roots of transformed plants was examined using substrate 5-bromo-4-choloro-3-indolyl β-D-glucuronide (X-Glu, cyclohexylammonium salt, Gold Biotechnology, USA) described by Jefferson et al. (1987).

3.2.6 Germination test of R1 progeny for hygromycin resistance

Seeds collected from the selfed progeny (R1 generation) of primary transformed plants were dehusked, surfaced sterilized, and placed on RT medium at 26 °C in darkness for 3 days. Only germinating seeds were transferred onto RT-H50 medium, cultured under a 16 h photoperiod at 26 °C for 7 days, and scored for hygromycin resistance. Those that vigorously formed roots and healthy shoots were regarded as hygromycin resistant. R1 seedlings from 3 primary transformants were further transferred to the greenhouse and grown to maturity.

3.2.7 Germination test of R2 progeny for hygromycin resistance and GUS expression

Seeds from 21 selfed R1 plants were germinated to form R2 progeny. Hygromycin resistance and GUS expression were tested and scored as described above. The hygromycin-resistant, GUS positive seedlings were transferred to the greenhouse. After 3 wks, plants were transplanted on 24 June, 1999 to the field at the LSU Rice Research Station, Crowley, LA.

3.2.8 Analysis of major agronomic traits of field-grown R2 progeny

Five major agronomic traits including plant height, number of tillers per plant, panicle length, number of spikelets per panicle, and fecundity were evaluated in mature plants. Plant height was measured from the soil surface to the tip of the leaf on the main stem of each plant. Number of tillers per plant was averaged from 3 randomly chosen plants. Panicle length and spikelets number per panicle were averaged from at least 3 randomly chosen panicles from different plants. Fecundity was expressed as the percentage of mature seeds over total spikelets of at least 3 randomly chosen panicles from different plants. Means and standard deviation were calculated.

3.2.9 Southern hybridization analysis

Genomic DNAs were extracted from leaf tissues according to Dellaporta et al. (1984). Purified DNAs for each sample were digested with *Hind* III and *BamH* I separately, electrophoresed in 0.8 % (w/v) agarose gels, transferred onto Hybond-NX (Amersham) membrane, and fixed using a UV Crosslinker (FB-UVXL-1000, FisherBiotech) set to deliver an energy dosage of 700 J m^{-2} as recommended by the manufacturer. Membranes were prehybridized at 65 °C for 2 hours in a buffer containing 6x SSC, 0.5% (w/v) SDS, 5 x Denhardt's solution, and 100 mg L^{-1} sheared, denatured salmon sperm DNA, and then hybridized at 65 °C overnight in the same buffer containing probes labeled with [α-^{32}P] dCTP (3,000 Ci/mol, Amersham) using random primed DNA labeling procedures (Feinberg and Vogelstein, 1983; Boehringer-Mannheim). A 1.1 kb *BamH*I fragment from pTRA151 (Fig. 2.1) was used as a probe for *hph*. After hybridization, membranes were washed (Sambrook et al,1989) and exposed to Kodak

BioMax MS autoradiography films with a Kodak BioMax MS intensifying screen at -80 °C for 16 hours.

3.3 Results and Discussion

3.3.1 Production of hygromycin-resistant, GUS-expressing plants from scutellum-derived calli inoculated with *A. tumefaciens* LBA4404 (pTOK233)

The first successful transformation of *japonica* (Hiei et al., 1994), *javanica* (Dong et al., 1996) subspecies of rice by *Agrobacterium tumefaciens* convincingly demonstrated that calli induced from scutella were suitable starting materials and *Agrobacterium tumefaciens* LBA 4404 (pTOK233) was the most effective strain in producing transgenic plants from all the cultivars tested. However, the protocol described by Hiei et al. included the use of a series of N6-derived media for calli induction, selection, and regeneration. In our preliminary experiments in transformation of one long-grain, U.S. cultivar Cypress and even the model *japonic* genotype Taipei 309, no transgenics were obtained even though the step-by-step protocol of Hiei et al. (1994) and Dong et al. (1996) were followed (data not shown), indicating that an optimized protocol must be established for the given genotypes. Based on our successful experience in bombardment-mediated transformation of U.S. rice lines, we adopted the media for scutellum-derived calli induction and regeneration used in the bombardment transformation and stringent selection using hygromycin at 50 mg L^{-1} rate in *Agrobacterium*-mediated transformation of scutellum-derived calli of two elite U.S. lines, LA9502065 and Cocodrie. In addition, factors influencing transformation frequency such as age, size, and type of rice cells as target tissues, culture medium, and the inoculum density of *Agrobacteria* were studied.

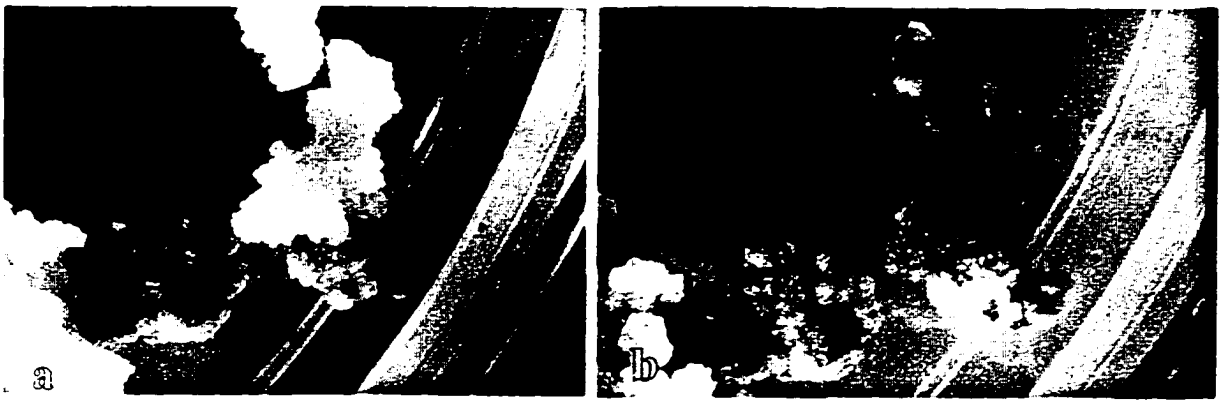

Fig.3.2. Proliferation of hygromycin-resistant, GUS-expressing calli on the CI-CH50 selection medium before (a) and after (b) GUS assay. The brown tissues were untransformed calli; light yellow tissues were transformed calli and stained by blue after GUS histochemical assay. x10.

A total of 1090 calli derived from scutella of LA9502065 and 785 calli of Cocodrie that have been co-cultivated with *A. tumefaciens* LBA4404 (pTOK233) were immediately cultured on CI-CH50 medium containing hygromycin. After two weeks of exposure to hygromycin, the majority of calli exhibited slow growth. To effectively eliminate non-transformed cells and recover stable transformants, calli were further transferred onto fresh CI-CH50 medium. After approximately four weeks of selection, proliferation of cells on or beside necrotic non-transformed calli were observed on the second selective medium (Fig. 3.2a). These proliferating, hygromycin-resistant cells/calli showed evidence of uniform expression of GUS (Fig. 3.2b), indicating they were transformed rather than mutants under the pressure of hygromycin selection. Plants were readily regenerated when the resistant calli were promptly transferred to

Fig.3.3. GUS expression in leaf and root segments of a transgenic R0 plant derived from LA9502065 transformed by *Agrobacterium tumefaciens* LBA4404. (a) leaf segment of a transgenic plant was stained blue (left) and no GUS expression was detected in the leaf segment of an unformed plant. (b) roots of the transgenic plant exhibited strong GUS activity (left) while no GUS expression was detected in roots of the untransformed plant.

regeneration medium PR1-H50 or PR2-H50 that contained hygromycin. GUS assays of segments of leaves or roots form regenerated plants displayed blue staining of the entire leaf and root segments (Fig. 3.3 a and b). The number of calli inoculated, transformed, and transformation frequency are given in Table 3.2. The overall transformation frequency of scutellum-derived calli for LA9502065 and Cocodrie were 10% and 11%, respectively. A total of 106 green plants for LA9502065 and 52 plants for Cocodrie were obtained. Our results substantiated the claims of Hiei et al. (1994) and Dong et al.(1996) that scutellum-derived calli were suitable targets for transformation mediated by *Agrobacterium* and also demonstrated that *Agrobacterium tumefaciens* LBA4404 containing the 'super-binary' plasmid pTOK233 is an effective strain for transformation of the long-grain U.S. cultivar Cocodrie and medium-grain line LA9502065.

3.3.2 Production of hygromycin-resistant, GUS-expressing plants from anther-derived calli inoculated with *A. tumefaciens* LBA4404 (pTOK233)

Various tissues from rice, namely, shoot apices, segments of roots from young seedlings, scutella or calli induced form scutella (Hiei et al., 1994; Dong et al., 1996), and immature embryos (Aldemita and Hodges, 1996) that have been tested or used for production of transgenic rice plants via *Agrobacterium*-mediated transformation are of somatic or diploid origin. Competence of calli derived from anthers or microspores that are of a haploid gametic source has not been explored. Theoretically, transformation of haploid tissues or cells permits a higher transformation efficiency due to the absence of dominant-to-recessive suppression and rapid recovery of diploid primary regenerants bearing a homozygous transgene after spontaneous or artificial doubling of chromosome (Chair et al., 1996). To investigate this hypothesis, a total of 716 calli of LA9502065 and 606 calli of Cocodrie were inoculated with *Agrobacterium tumefaciens* LBA4404 (pTOK233) and subjected to hygromycin selection under the same conditions. As a result, a higher transformation frequency (15%) was obtained with anther-derived calli from both LA9502065 (105/716) and Cocodrie (94/606), compared with those of scutellum-derived calli (10-11%). Plant regeneration was also obtained from those presumably haploid, hygromycin-resistant, and GUS-expressing calli (Table3.2). However, genotypic influence on transformation frequency was observed among 4 R_1 anther cultures and one R_2 line. The transformation frequency of anther-derived calli varied from 1% (ACF126F2) to 28% (ACF222). In terms of plant regeneration, albinism severely reduced the recovery of green transgenic rice plants. At best only 33% (20/61 for LA9502065) and

Table 3.2. Number of calli inoculated, calli transformed by *Agrobacterium tumefaciens* LBA4404 (pTOK233), and plants regenerated with HygR and GUS expression.

Genotype	Explant	Exp. no.	No. of calli inoculated	No. of HygR, GUS+ calli†	Transform-ation frequency	No. of HygR, GUS+ plants		
						Green	Albino	Total
LA9502065	Scutellum	1	60	11	0.183	10	0	10
		2	127	34	0.268	24	0	24
		3	100	14	0.14	14	0	14
		4	257	23	0.089	22	0	22
		5	282	12	0.043	12	0	12
		6	128	18	0.141	18	0	18
		7	219	8	0.037	6	1	7
		Total	1090	120	0.102	106	1	107
	Anther	1	112	25	0.223	3	10	13
		2	126	23	0.183	12	6	18
		3	151	15	0.099	5	12	17
		4	182	26	0.143	0	6	6
		5	145	16	0.11	0	7	7
		Total	716	105	0.147	20	41	61
								0
Cocodrie	Scutellum	1	129	11	0.085	5	0	5
		2	248	20	0.081	8	0	8
		3	124	13	0.105	11	0	11
		4	156	24	0.154	20	0	20
		5	128	21	0.164	8	0	8
		Total	785	89	0.113	52	0	52
	Anther	1	122	12	0.098	9	2	11
		2	120	15	0.125	2	3	5
		3	124	25	0.202	0	12	12
		4	240	42	0.175	0	16	16
		Total	606	94	0.155	11	33	44
ACF157	Anther	1	128	8	0.063	2	6	8
		2	66	2	0.030	0	2	2
ACF179	Anther	1	140	19	0.136	0	5	5
		2	149	10	0.067	0	0	0
ACF186	Anther	1	156	9	0.058	0	3	3
ACF222	Anther	1	68	19	0.279	0	4	4
ACF126F2	Anther	1	156	1	0.006	0	0	0

† HygR, resistance to hygromycin at 50 mg L^{-1}; GUS+, cells appeared blue when treated with X-Gluc substrate.

25 % (11/44 for Cocodrie) of regenerated plants were green plants, whereas in the worst case, no green plant was obtained for ACF179, ACF182, and ACF222. In addition, the majority of anther-derived plants grown in the greenhouse were sterile, which made the genetic study of transgenes impossible. The present study, however, for the first time demonstrated that anther-derived calli are competent for *Agrobacterium*-mediated transformation. Problems of albinism and sterility of regenerated plants should be addressed in future studies to utilize the potential advantages of anther-derived calli as target tissues in routine transformation work.

3.3.3 Factors influencing the efficient transformation of scutellum calli

It is clear that numerous factors are of critical importance in the *Agrobacterium*-mediated transformation of rice and this multiplicity of factors probably explains why it was initially so difficult to apply this technology to rice (Hiei et al., 1997). The effect on transformation frequency of genotype and tissue types, e.g., calli derived from scutella vs. anther, has been showed in this study (Table 3.2). Apart from those given factors, choice of rice tissues at the right physiological state associated with cell age, the size of target tissues, and bacterial virulence influenced by the culture medium and the density of inoculum should be optimized.

3.3.3.1 Cell age and target size

Calli 4-, 8-, 12-, and 16- week-old were tested in the transformation experiment. Transgenic calli and plants were obtained from 2-4 mm size calli of all ages tested but few or no transgenics were recovered from calli of smaller (1-2 mm), larger (> 4 mm), or from suspension cultures (<2 mm) (Table 3.3). These results indicated callus size rather

Table 3.3. Effect of cell age and calli size on transformation efficiency (LA9502065).

Cell age (wk)	Cell type †	Calli size (mm)	No. of calli inoculated (A) ‡	No. of HygR, GUS+ calli (B)	Transform-ation frequency (A/B)	No. of HygR, GUS+ plants (C)	Transform-ation efficiency (C/A)
4	Calli	1-2	45	2	0.044	2	0.044
	Calli	2-4	47	16	0.340	15	0.319
	Calli	>4	45	1	0.022	0	0.000
8	Calli	1-2	57	0	0.000	-	-
	Calli	2-4	54	9	0.1667	12	0.267
	Calli	>4	52	0	0.000	0	-
	Susp.	<2	-	2	-	1	-
12	Calli	1-2	45	0	0.000	-	-
	Calli	2-4	46	12	0.261	12	0.261
	Calli	>4	45	2	0.044	2	0.044
	Susp.	<2	-	0	-	0	-
16	Calli	1-2	42	0	0.000	-	-
	Calli	2-4	48	10	0.208	14	0.292
	Calli	>4	45	2	0.044	1	0.022
	Susp.	<2	-	0	-	-	-

† Calli and suspension cultures (Susp.) were derived from scutella.
‡ A '-' means not applicable.

than cell age is critical. Calli of 2-4 mm in size were chosen due to their rapid proliferation and embryogenic potential. The smaller calli or suspension cultures were not suitable targets for the transformation and selection since they turned necrotic readily due to the physical damage caused by the bacterial washing procedure after co-cultivation. Conversely, target tissues larger than 4 mm displayed undesirable tolerance to hygromycin and made complete elimination of untransformed cells difficult due to a 'cross-protection' phenomena in which untransformed cells on the top of large tissues were not sufficiently exposed to hygromycin.

3.3.3.2 Composition of medium for *Agrobacterium* growth and the density of inoculum

AB medium was specially designed for the growth of *Agrobacterium tumefaciens* and bacteriophage (Chilton et al., 1974). It has been used virtually in all *Agrobacterium*-mediated transformation of rice except for one report in which LB medium (Miller, 1972) was used for the growth of *Agrobacterium tumefaciens* strains LBA4404 and AGL (Wang et al, 1997). AB medium is composed of inorganic nutrients supplemented with glucose which was important in the induction of *Agrobacterium* virulence genes synergistically with the phenolic inducer acetosyringone (Cangelosi et al., 1990). Conversely, LB medium is composed of organic components supplemented with sodium chloride as an osmotic requirement for the growth of *Escherichia coli* cells. In a comparative test, both media were used for preparation of *Agrobacterium tumefaciens* LBA4404 (pTOK233) as inoculum, As a result, rice tissues were transformed by Agrobacteria, regardless of the choice of medium (Table 3.4). However, the inoculum density affected the transformation frequency significantly. In a range of 0.5-2.0x10^9 cells ml^{-1}, the optimal density of inoculum for the highest transformation frequency was 1.5 x10^9 cells ml^{-1} with the use of AB medium (27%) and 2.0 x 10^9 cells ml^{-1} with LB medium (11%). Transformation frequency dropped dramatically with the reduced density of inoculum (1.0x10^9 cells ml^{-1}). Zero to 1 % of target tissues were transformed at the lowest inoculum density tested of 0.5 x10^9 cells ml^{-1}. Therefore, careful preparation of *Agrobacterium* inoculum at a optimal density for a given medium is of critical importance for a routine, reproducible rice transformation system mediated by *Agrobacterium*. Similar findings were observed

Table 3.4. Effect of culture medium of *Agrobacterium* and the density of inoculum on transformation frequency of rice scutellum-derived calli (LA9502065).

Medium	OD_{595}	Inoculum density (cells ml^{-1}) †	No. of calli inoculated (A)	No. of HygR, GUS+ calli (B)	Transformation frequency (A/B)
AB	2.0	$2.0x10^9$	54	10	0.185
	1.5	$1.5x10^9$	67	18	0.269
	1.0	$1.0x10^9$	84	7	0.083
	0.5	$0.5x10^9$	82	0	0.000
LB	2.0	$2.0x10^9$	74	8	0.108
	1.5	$1.5x10^9$	86	7	0.081
	1.0	$1.0x10^9$	80	4	0.050
	0.5	$0.5x10^9$	75	1	0.013

† *Agrobacterium* cells were collected from AB or LB plates and then suspended in AAM medium; Readings were estimated based on 0.1 OD_{595} being roughly equivalent to 10^8 cells ml^{-1} (Ausubel et al., 1992).

in *Agrobacterium*-mediated transformation of maize (Ishida et al., 1996) and barley (Wu et al., 1998).

3.3.4 Characterization of transgenic plants in R0 and R1 generations

A total of 158 independent, hygromycin-resistant, GUS-positive plants of LA9502065 (106) and Cocodrie (52) from scutellum-derived calli infected with LBA4404 (pTOK233) were grown in a greenhouse in the Fall of 1998. Virtually all plants were morphologically normal, but a majority (90 %) produced few or no seeds. Reasons for the sterility could be classified as genetic factors associated with the transformation and tissue culture or merely environmental conditions. Unfavorable greenhouse conditions and severe sheath rot (*Sarocladium oryzae*) disease and rice stink bug (*Oebalus pugnax*) infestation were apparently major factors responsible for sterility. This was evidenced by the fact that R1 progeny of poor seed-setting transgenic plants

produced as many seeds as untransformed parental plants by self-pollination in Spring 1999.

A total of 33 independent, hygromycin-resistant, GUS-positive plants of LA9502065 (20), Cocodrie (11), and ACF157 (2) from anther-derived calli infected with LBA4404 (pTOK233) were grown in a greenhouse under the same environmental conditions as in Fall 1998. A majority (79%) of the plants exhibited morphological aberrations, such as dwarfism and twisted panicles. In terms of fertility, complete to partial fertility was observed. One reason for sterility could be the haploid nature of some plants that had not undergone the spontaneous chromosomal doubling.

3.3.5 Southern blot hybridization analysis

Integration of the introduced *hph* gene into the rice genome was confirmed by Southern blot hybridization using the 1.1 kb fragment of pTRA151 (Zheng et al., 1991) as *hph* probe. Genomic DNA of R0 plants representative of 3 independent lines (804-2, 826-3, and 826-4) and their R1 progeny plants were digested with *Hind* III and *BamH* I restriction enzymes As shown in Fig 3.1, digestion of the T-DNA region of pTOK233 with *Hind* III conveniently cleaves the DNA into three fragments. The center fragment corresponds to a 3.1 kb region that covers the 35S-IG-NOS gene cassette, while the other fragments extend beyond the right and left T-DNA borders. However, there is no *Hind* III restriction site in the fragment containing *hph* gene within the left border. It was well documented that T-DNA integrates into plant genome with defined ends of left and right 25-bp border repeats (Hiei et al., 1994). As a result, hybridization of *hph* probe to genomic DNA digested with *Hind* III permits an estimation of independent integration

events based on the size/number of hybridization bands. Two bands were detected in Line 804-2 and its progeny, whereas only one band was found in Lines 826-3, 826-4 and their progeny (data not shown). The left border analysis indicated the presence of two T-DNA inserts for Line 804-2 with its progeny and a single copy for Lines 826-3, 826-4, and their progeny. In contrast, digestion of T-DNA with *BamH* I releases only a 1.1 kb fragment covering the *hph* coding sequence. It was found in this study that hybridization of *hph* probe to genomic DNA digested with *BamH* I revealed only a common band sized 1.1 kb among all transgenic rice lines (804-2, 826-3, and 826-4) and their R1 progeny, whereas no band was present for the wild type (data not shown). This further demonstrated that the transgene in T-DNA of pTOK233 was integrated into rice genome via *Agrobacterium*-mediated transformation.

3.3.6 Inheritance of the *hph* and GUS gene

3.3.6.1 Segregation of hygromycin resistance in R1 and R2 generations

The selfed progeny (R1 and R2 generations) were evaluated for resistance to hygromycin and GUS expression. The patterns of segregation for hygromycin resistance of 3 independent R1 lines and 21 R2 lines were shown in Table 3.5. Resistant and sensitive seedlings were clearly distinguishable on the rooting medium that contained hygromycin. A segregation ratio of 9:7 was observed for Line 804-2 and its progeny lines, indicating existence of two partial dominant, but complementary loci in the rice genome. This was consistent with the insert copy number (2) estimated by Southern blot analysis for this line. A segregation ratio of 3:1 was observed in Line 826-3 and its R2 generations, indicating the *hph* gene was transmitted to its progeny as a single dominant

Table 3.5. Segregation for hygromycin resistance in R1 and R2 transgenic LA9502065 lines and GUS expression in HgyR progeny.

Generation	Line	No. of seedlings tested	No. of HygR seedlings	No. of HygS seedlings	X^2 ratio†	X^2 value‡	No. of HygR seedlings assayed for GUS	No. of GUS+ seedlings
R1	804-2	11	7	4	9:7	0.244	7	7
R2	804-2-1	36	20	16	9:7	0.007	20	20
	804-2-2	17	11	6	9:7	0.494	11	11
	804-2-3	46	33	13	3:1	0.261	33	33
	804-2-4	32	1	31	-	-	1	1
	804-2-5	15	9	6	9:7	0.086	9	9
	804-2-7	35	28	7	3:1	0.467	11	11
R1	826-3	9	6	3	3:1	0.333	6	6
R2	826-3-1	35	30	5	3:1	2.143	24	24
	826-3-2	37	27	10	3:1	0.081	18	18
	826-3-4	37	25	12	3:1	1.090	17	17
	826-3-6	33	27	6	3:1	0.818	11	11
R1	826-4	15	12	3	3:1	0.200	3	3
R2	826-4-1	36	30	6	3:1	1.333	21	21
	826-4-2	37	37	0	stable	-	16	16
	826-4-3	39	33	6	3:1	1.923	22	22
	826-4-4	30	29	0	stable	-	5	5
	826-4-5	34	34	0	stable	-	8	8
	826-4-6	33	21	12	9:7	0.732	5	5
	826-4-7	32	19	13	9:7	0.127	12	12
	826-4-8	32	24	8	3:1	0.000	20	20
	826-4-9	34	20	14	9:7	0.092	10	10
	826-4-10	36	24	12	3:1	1.333	11	11
	826-4-11	33	19	14	9:7	0.024	7	7
Total							308	308

† Best Chi square ratio of those tested; A '-' indicates segregation did not fit any known ratio. 'stable' indicates the corresponding line is homozygous to *hph* gene.

‡ df=1, X^2=3.841 at the 0.05 probability level.

gene. The segregation ratio of Line 826-4 and its progeny (4/11) also fit a 3:1 model for single dominant gene inheritance, however, an abnormal ratio (9:7) was also observed in

4 R2 lines. In these cases it seemed likely that the *hph* gene expression was being silenced in some R2 progeny.

3.3.6.2 Gene silencing in R2 progeny of the transgenic line 804-2

To investigate co-expression of the linked GUS gene in R1 and R2 generations, all hygromycin-resistant seedlings were tested for GUS gene expression. All (308/308) hygromycin-resistant plants were GUS positive (Table 3.5), indicating *hph* was a reliable selection marker gene for the recovery of its-linked transgene co-integrated into the rice genome via *Agrobacterium*-mediated transformation. However, in another case study of co-expression of the *hph* and GUS genes in transgenic rice line 804-2, preferential silencing of the *hph* gene expression was observed in R2 progeny. That was observed by GUS expression in hygromycin-sensitive seedling (Fig. 3.4). In a total of 146 seedlings

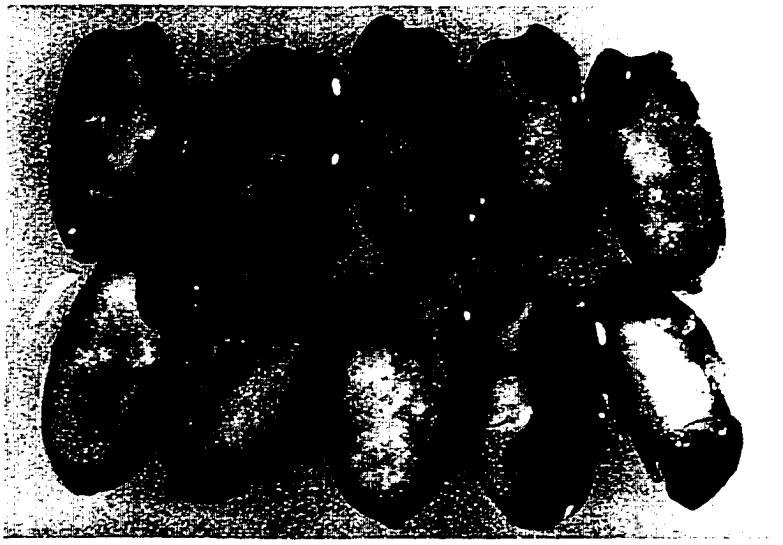

Fig.3.4. GUS expression in hygromycin-sensitive seeds of R2 progeny of Line 804-2 derived from LA9502065 transformed by *Agrobacterium tumefaciens* LBA4404 (pTOK233), indicating preferential silencing of the *hph* gene expression.

Table 3.6. Silencing of hygromycin-resistance gene (*hph*) expression in R2 progeny of the transgenic line 804-2 derived from LA9502065.

Line	HygR† GUS+	HygR GUS-	HygS GUS+	HygS GUS-	No. of seedlings tested
804-2-1	20	0	2	14	36
804-2-2	11	0	2	4	17
804-2-3	33	0	1	12	46
804-2-4	1	0	31	0	32
804-2-5	9	0	3	3	15
Total	74	0	39	33	146

† HygR, hygromycin-resistant; HygS, hygromycin-sensitive; GUS+, expressing GUS; GUS-, not expressing GUS.

tested, apart from 74 hygromycin-resistant seedlings being GUS-positive as expected, 27% (39/146) seedlings were found hygromycin-sensitive but GUS-positive (Table 3.6), indicating expression of the *hph* gene was silenced. Transgenic rice lines with high copy number of the *hph* gene were found prone to gene silencing (Wang et al., 1997). That was more or less substantiated by the present study since two loci containing at least two insert copies of *hph* gene were present in transgenic line 804-2, based on Southern blot analysis and segregation of hygromycin resistance in R1 and R2 generation (Table 3.6). Methylation of cytosine residues was believed responsible for transgene silencing because silenced transgenes could be reactivated by treatment with 5-azacytidine, an inhibitor of methylation of cytosine (Kumpatla et al., 1997; Kohli et al., 1999). Moreover, Kohli et al.(1999) reported that methylation of cytosines may not spread to adjacent regions, hence other transgenes in the vicinity of the silenced transgene remain active. This was consistent with results observed in the present case study.

3.3.7 Field evaluation of transgenic rice plants in the R2 generation

Five major agronomic traits of 92 progeny plants from 16 R2 lines were evaluated and compared with those of wild-type parental plants (Table 3.7). The results demonstrated that *Agrobacterium*-transformed rice plants exhibited normal phenotypes including fertility in the field. One R2 line 804-2-1 showed low plant height (66.0 cm), short panicle length (17.0 cm), and low number of spikelets per panicle (111.8). However, this dwarf transgenic line had a normal number of tillers (14.7) and set seeds

Table 3.7. Mean values for height, tillers per plant, panicle length, spikelets per panicle, and fecundity for wild-type and 16 transgenic lines derived from *Agrobacterium* transformation, June 24-September 3, 1999, Rice Research Station, Crowley, LA.

Line	No. of plants examined	Height† (cm)	No. of tillers per plant	Panicle length (cm)	No. of spikelets per panicle	Fecundity (%) ‡
Wild-type	3	84.3 (3.21)	11.7 (1.53)	23.7 (3.36)	164.0 (18.25)	14.0 (3.32)
804-2-1	10	66.0 (2.05)	14.7 (4.14)	17.0 (0.93)	111.8 (24.39)	35.2 (13.80)
826-3-1	11	89.0 (4.07)	12.0 (1.00)	23.3 (1.52)	220.0 (17.95)	32.7 (11.34)
826-3-2	5	90.4 (5.17)	12.0 (2.64)	23.4 (2.63)	282.4 (17.33)	42.7 (17.35)
826-3-4	4	97.5 (3.69)	13.3 (1.52)	22.5 (1.25)	233.0 (31.14)	46.1 (3.22)
826-3-6	5	98.4 (3.78)	23.0 (6.24)	21.8 (1.64)	224.2 (43.28)	61.0 (6.22)
826-4-1	4	82.8 (2.75)	10.0 (1.00)	19.3 (1.44)	181.8 (16.92)	55.3 (16.00)
826-4-2	11	87.4 (1.50)	12.7 (1.53)	18.8 (1.95)	185.2 (61.85)	42.6 (21.46)
826-4-3	3	98.0 (2.64)	15.0 (2.64)	23.1 (0.85)	226.0 (22.00)	24.7 (9.39)
826-4-4	10	94.9 (3.57)	15.3 (3.06)	23.7 (2.32)	219.4 (28.01)	57.4 (6.90)
826-4-5	11	90.4 (6.85)	16.3 (2.08)	21.9 (0.22)	243.3 (31.17)	40.7 (13.90)
826-4-6	3	97.0 (1.73)	16.0 (3.61)	26.4 (1.87)	244.7 (22.74)	28.5 (19.20)
826-4-7	3	89.7 (2.31)	12.0 (1.73)	23.4 (2.21)	195.3 (68.57)	19.2 (7.40)
826-4-8	3	92.0 (2.00)	7.7 (2.08)	24.4 (1.96)	198.0 (22.65)	45.7 (5.23)
826-4-9	3	94.7 (208)	13.7 (3.21)	22.7 (1.04)	215.7 (20.03)	37.9 (9.53)
826-4-10	3	90.7 (8.50)	9.0 (1.73)	23.7 (2.52)	255.7 (21.93)	46.1 (19.74)
826-4-11	3	90.0 (2.00)	11.3 (3.05)	23.5 (1.70)	180.3 (35.80)	33.6 (3.23)

† Mean of individual line; Standard deviation are indicated in parentheses.
‡ Fecundity was calculated as percentage of fertile seeds in the whole panicle.

normally. Four R2 lines of 826-3 and 11 R2 lines of 826-4 showed higher plant height (87.4-98.4 cm) and larger numbers of spikelets per panicle (180-280) that contrasted favorably with low plant height (84.3 cm) and small number of spikelets per panicle (164) for the wild-type plants. Fecundity of the transgenic rice lines were also satisfactorily higher than that of the wild-type plants under the given field conditions. No sterile plants were observed. Test plots should be conducted in the future to further evaluate field performance of these transgenic rice lines.

CHAPTER 4. PRODUCTION OF GLUFOSINATE-RESISTANT RICE VIA PARTICLE BOMBARDMENT OF HAPLOID, ANTHER-DERIVED CALLI AND CELL SUSPENSIONS

4.1 Introduction

Production of transgenic rice (*Oryza sativa*) has been reported by various researchers using particle bombardment technology (see review by Christou, 1997). However, in most rice transformation reports, the target cells were immature embryos (Christou et al., 1991; Cooley et al., 1995; Li et al., 1993), immature embryo-derived calli (Wu et al., 1997), mature seed-derived calli (Chen et al., 1998; Ghosh Biswas et al., 1998; Sivamani et al., 1996) or suspension cells (Cao et al., 1992; Zhang et al., 1996) which were of somatic or diploid origin. Virtually all of these cultured cells carry two or more copies of their genes which may reduce efficiency for the expression of engineered traits. In addition, segregation of transgenes in the progeny of primary transformants, regardless of its pattern of Mendelian or non-Mendelian inheritance, makes immediate recovery of homozygous transgenic plants impossible. Conversely, transformation of haploid cells of anther or microspore origin has the theoretical advantage of permitting a higher efficiency for the expression of transgenes and rapid recovery after chromosome doubling of haploid primary regenerants bearing a homozygous transgene (Chair et al., 1996).

Production of transgenic plants from bombardment of microspores, pollens, microspore-derived embryos or anther-derived calli have been reported in canola (*Brassica napus*) (Chen and Beversdorf, 1994), wheat (*Triticum aestivum*) (Jahne et al., 1994), *Nicotiana rustica* (Nishihara et al.,1995; Stoger et al., 1995), maize (*Zea mays*)

(Wan et al.,1995), barley (*Hordeum vulgare*) (Wan and Lemaux, 1994; Yao et al., 1997), and rapeseed (*Brassica napus*) (Fukuoka et al., 1998). The transformation of microspore-derived cell suspension protoplasts of rice by polyethylene glycol (PEG) mediated gene transfer produced diploid transgenic plants, and both homozygous and hemizygous integration of transgenes were confirmed (Chair et al.,1996). The competence of anther-derived embryogenic calli for *Agrobacterium*-mediated transformation were also demonstrated in our previous studies (unpublished data). However, there is no report to date of production of transgenic rice plants via particle bombardment of haploid, anther- or microspore-derived cells as target tissues. The objectives of the present study are to evaluate competence of anther-derived cultures as target tissues for particle bombardment-mediated transformation, and to simultaneously develop herbicide glufosinate-resistant transgenic rice plants for elite U.S. breeding lines.

4.2 Materials and Methods

4.2.1 Rice genotypes and culture media

Media used for tissue culture and transformation are listed in Table 4.1. Seven long-grain breeding lines, namely, LA9502002, LA9502008, 96URN048, 96URN082, 96URN085, 96URN091, and 96URN115, three medium-grain lines, LA9502065, 96URN128, and 96URN131 (Table 4.2) were used for callus induction directly from anthers. Primary calli derived from anther culture of other lines, 7047H1, 7215H1, 1088, and Ti304H1 and anther-derived cell suspensions of 7845, 7631 were supplied by Dr. Qiren Chu, the Rice Biotechnology Laboratory of the LSU Rice Research Station, Crowley, LA.

Table 4.1. Composition of media for rice anther culture and transformation in this study.

Medium	Composition
CI	CC salts and vitamins (Potrykus et al. 1979), 20 g L^{-1} sucrose, 18.2 g L^{-1} mannitol, 18.2 g L^{-1} sorbitol, 2 mg L^{-1} 2,4-D, 7 g L^{-1} agarose (Type I-A, Sigma), pH 5.8
CHU	Chu salts and vitamins (Chu et al., 1997), 20 g L^{-1} sucrose, 20 g L^{-1} sorbitol, 20 g L^{-1} maltose, 1 mg L^{-1} 2,4-D, , 0.1 mg L^{-1} zeatin, 2 g L^{-1} phytagel (Sigma), pH 5.8
R203	R2 salts and vitamins (Ohira et al., 1973), 20 g L^{-1} sucrose, 10 g L^{-1} maltose, 560 mg L^{-1} proline, 1 mg L^{-1} 2,4-D, pH5.8
CHU-H50	CHU with 50 mg L^{-1} hygromycin B (Boehringer Mannheim), pH 5.8
PR2-H50	MS salts and vitamins (Murashige and Skoog,1962), 20 g L^{-1} sucrose, 10 g L^{-1} maltose, 2 mg L^{-1} kinetin, 0.5 mg L^{-1} NAA, 7 g L^{-1} agarose, 50 mg L^{-1} hygromycin B, pH 5.8
RT	MS salts and vitamins (Murashige and Skoog, 1962), 20 g L^{-1} sucrose, 2 g L^{-1} Phytagel (Sigma), pH 5.8
RT-H50	RT with 50 mg L^{-1} hygromycin B, pH 5.8
RT-B4	RT with 4 mg L^{-1} bialaphos, pH 5.8

4.2.2 Calli and suspension cultures derived from anthers

Panicles from rice plants grown at the LSU Rice Research Station at Crowley, LA were picked at the booting stage when microspores were at the mid-to-late uninucleate stage (the flag leaf node has emerged approximately 0.5 to one inch). Panicles were harvested generally from 9 to11 AM. All except the top two leaves were removed from harvested panicles that were wrapped in moistened paper towels, placed in plastic bags, and subjected to cold pre-treatment at 4 °C for 7-10 days. The panicles were then surface-sterilized in 50% (v/v) commercial bleach (containing 6.25 % sodium hypochlorite) for 15 min. After removal of the remaining leaves around the panicles, spikelets were

dissected under sterile conditions and anthers were inoculated on CHU medium or CI medium (Table 4.1) for callus induction. After 5-6 weeks, calli of 1-1.5 mm in diameter were subcultured on fresh CHU medium every 3 wks or suspended and subcultured in R2 liquid medium on a rotary shaker (80 rpm). Calli or suspension cultures in the logarithmic phase of growth (3-4 days after subculture for 2 to 5 months) were tested for transformation experiments. Since the preparation of primary calli derived from anthers is rather season- and environment-dependent, as well as labor intensive, calli and cell suspension subcultured at a different times were used to study the effect of cell age of anther cultures on transformation efficiency.

4.2.3 Plasmid used for bombardment

Plasmid pPAT63 was used for bombardment of haploid targets in this experiments. pPAT63 is a derivative from the plasmid pB2/35SAcK (Hoechst Schering AgrEvo) which contains a synthetic *pat* gene (Eckes et al., 1989) encoding phosphinothricin acetyltransferase (PAT) that inactivates phosphinothricin (PPT), active ingredient of the herbicide glufosinate (trade names Liberty, Basta, Ignite, Finale). To facilitate selection of transformants with hygromycin B, a 1.7 kb *Hin*d III fragment containing a mutated version of the *hph* gene from plasmid pTRA151 (Zheng et al.1991) was cloned into a unique *Hin*d III site of pB2/35AcK by the author. The size of pPAT63 is 5.8 kb.

4.2.4 Optimization of particle bombardment

The helium-driven Biolistic PDS-1000/He (Bio-Rad) system was used in all experiments. Plasmid DNAs were coated onto gold particles (1 μm in size) according to

Sanford et al.(1992). For anther-derived calli, approximately 30 embryogenic calli (2-3 mm in diameter) were placed evenly into a 100 x 15 mm plastic petri dish containing 25 ml of fresh CHU medium one day prior to bombardment. For routine bombardment, the distance from repture disc to macro-carrier, from macro-carrier to stopping screen, and from stopping screen to target tissues was 1, 1, and 9 cm, respectively. The rupture disc strength was 1,100 psi and vacuum pressure of the bombardment chamber was 0.09 MPa (27 in Hg). Bombardment was conducted once per petri dish. For cell suspensions, the effect of bombardment distance from the stop screen to target plate (6, 9, and 13 cm) on transformation frequency was tested.

4.2.5 Osmotic treatment of cell suspensions

Due to the distinct characteristics of cell suspensions, such as small size and active growth, two different methods for osmotic treatment prior to bombardment were carried out as follows. For the first method, cell suspensions were collected with a spatula and spread evenly onto one piece of dry sterilized 5-cm Whatman #4 filters (Whatman Laboratory Division, England) in an empty Petri dish. The dishes were sealed with parafilm and kept at 25 °C for 6, 12, or 24 hours. For the second method, cell suspensions were plated onto a filter pre-set on 1.0% agarose-solidified CHU medium and cultured for 2, 7, or 14 days prior to bombardment. Partially dehydrated or pre-cultured cells were transferred onto the center of 100 x 15 mm Petri dishes containing 25 ml of CHU medium and bombarded once per dish.

4.2.6 Selection and regeneration of transformants

One day after bombardment, embryogenic calli or filters carrying cell suspensions were transferred to CHU-H50 medium supplemented with hygromycin B (Boehringer Mannheim). Calli or cell suspensions set on filters were subcultured on the same fresh selection medium at three weeks intervals. After approximately 6 wks of selection, hygromycin-resistant calli were recovered among necrotic untransformed cells and transferred to PR2-H50 regeneration medium (Table 4.1). In approximately 2 wks, shoots or plantlets were recovered and transferred into Magenta boxes (Sigma) containing RT-B4 rooting medium (Table 4.1). Plantlets 7-10 cm high with vigorous root development were transferred to potting soil in a greenhouse and designated as R0 plants.

4.2.7 Glufosinate application to R0 plants in the greenhouse

Putative transgenic rice plants grown to the third- or fourth leaf stage in the greenhouse were tested for response to glufosinate herbicide (200 g L^{-1} PPT, Hoechst AG, Germany) by spraying with a 1.0 % (v/v) solution plus 0.1 % (v/v) Tween 20. The actual concentration of PPT used was 2000 mg L^{-1}. Resistant and susceptible plants were scored 7 DAT. Plants that survived were grown to maturity in the greenhouse.

4.2.8 Glufosinate application to R1 progeny in the greenhouse

Seeds collected from the selfed progeny (R1 generation) of 9 fertile, primarily transformed plants of LA9502065 and 4 fertile transgenic plants of 7845 were directly sown in potted soil in the greenhouse. After approximately 3 wks, plants at 3-4 leaf stage were sprayed with glufosinate at 2000 mg L^{-1}. Herbicide resistance was scored 7 DAT.

4.2.9 Southern hybridization analysis

Genomic DNAs were extracted from leaf tissues according to Dellaporta et al. (1984). Purified DNAs for each sample were digested with *EcoR* I, electrophoresed in 1.0 % (w/v) agarose gels, transferred onto Hybond-NX (Amersham) membrane, and fixed using a UV Crosslinker (FB-UVXL-1000, FisherBiotech) set to deliver an energy dosage of 700 J m^{-2} as recommended by the manufacturer. Membranes were prehybridized at 65 °C for 2 hours in a buffer containing 6x SSC, 0.5% (w/v) SDS, 5 x Denhardt's solution, and 100 mg L^{-1} sheared, denatured salmon sperm DNA, and then hybridized at 65 °C overnight in the same buffer containing probes labeled with [α-^{32}P] dCTP (3,000 Ci/mol, Amersham) using random primed DNA labeling procedures (Feinberg and Vogelstein, 1983 ; Boehringer-Mannheim). A 0.45 kb *Sma* I fragment from pPAT63 (Fig. 2.1) was used as a probe for *pat*. After hybridization, membranes were washed (Sambrook et al,1989) and exposed to Kodak BioMax MS autoradiography films with a Kodak BioMax MS intensifying screen at -80 °C for 16 hours.

4.3 Results and Discussion

4.3.1 Preparation of embryogenic calli and cell suspensions from anther cultures

One of the prerequisites for successful transformation is preparation of a large quantity of actively growing and regenerable target tissues. To exploit the potential of haploid anther cultures as target tissues for production of transgenic rice plants via particle bombardment, a total of 34,440 anthers from 10 U.S. rice lines were plated on CHU or CI medium for callus induction (Table 4.2). Anthers were cultured at 26 °C in the darkness. In approximately 35-40 days, a large quantity of embryogenic calli (1502

Table 4.2. Effect of medium on callus induction from anthers.

Genotype	Medium	No. of dishes	No. of anthers inoculated †	No. of calli obtained	Callus induction rate, %
LA9502002	CHU	13	1560	48	3.1
LA9502008	CHU	57	6840	164	2.4
	CI	6	720	1	0.1
96URN048	CHU	6	720	18	2.5
96URN082	CHU	39	4680	66	1.4
96URN085	CHU	6	720	27	3.7
	CI	2	240	0	0
96URN091	CHU	17	2040	18	0.9
	CI	2	240	0	0
96URN115	CHU	4	480	15	3.1
	CI	2	240	2	0.9
LA9502065	CHU	95	11400	855	7.5
	CI	10	1200	11	0.9
96URN128	CHU	4	480	41	8.5
	CI	2	240	0	0
96URN131	CHU	20	2400	252	10.5
	CI	2	240	0	0

† Number of anthers inoculated was the product of number of dishes and 120 anthers per dish.

calli/31,260 anthers) were obtained from anthers plated on CHU medium. The callus induction rate varied from 0.9% (96URN091, a long-grain line) to 10.5% (93IRN131, a medium-grain line), with an overage of 4.8%. In comparison, none or few calli (14 calli/3180 anthers) were induced from anthers plated on CI medium which was used for

production a large quantity of embryogenic calli from scutella in our previous experiments. These results indicated that choice of a suitable medium depending on the type of explants used is of critical importance in preparation of target tissues. CHU medium (Chu et al., 1997) was proved suitable for induction of embryogenic calli from anthers of U.S. rice lines.

Due to the season- and environment-dependence of anther sources, primary calli were further subcultured every three weeks on CHU medium for all ten lines tested. Calli of LA9502065 and LA9502008 were transferred to R203 liquid medium (Table 4.1) to initiate cell suspensions. Proliferation of cell clusters were obtained after four weeks of subculture in R203 medium. For long-term maintenance of embryogenic cells, cell suspensions were subcultured weekly.

4.3.2 Bombardment of calli and cell suspensions and selection of transformed cells

Embryogenic calli and cell suspensions placed on filters were bombarded with gold particles coated with plamsid DNA of pPAT63. The plasmid contained the *hph* gene and a synthetic *pat* gene conferring resistance to hygromycin antibiotic and glufosinate herbicide, respectively. Both genes were driven by the cauliflower mosaic virus (CaMV) 35S promoter. Based on our previous experiments in bombardment of scutellum-derived calli, selection for hygromycin resistance took at least two weeks less than selection for bialaphos. To reduce the selection period and possible somaclonal variation, hygromycin B was used as the selective agents. Bombarded calli or cell suspensions on filters were transferred and subcultured on selection medium containing 50 mg L^{-1} hygromycin. The growth of bombarded callus pieces and cell suspensions on the first selection medium

was severely inhibited. On the second selection medium, most untransformed callus pieces and cell suspensions showed necrosis, whereas transformed showed proliferating sectors. To eliminate the remaining untransformed cell clusters, the growing cells were isolated from the other bombardment explants and subjected to further selection on fresh hygromycin-containing medium. The majority of growing cells survived the stringent selection and proliferated to form hygromycin-resistant (HygR), embryogenic calli. The growth rates of the HygR calli on selection medium were similar to those of untransformed calli on non-selection medium. No HygR calli were observed to grow out of non-bombarded control cells after subject to the same selection pressure. These HygR calli were readily regenerated into plantlets in approximately 2-3 wks after transferred onto PR2-H50 medium. A comparative test revealed that the stringent selection as described above was essential for efficient recovery of stable transformants from bombarded cell suspensions, because few or one stable, HygR calli were obtained when selection for hygromycin resistance was delayed for longer than a week post-bombardment or if hygromycin concentration was reduced to 25 mg L^{-1} .

4.3.3. Osmotic enhancement of stable transformation of rice cell suspensions

In a pilot experiment (data not shown) with Line 7845, actively-growing cell suspensions without dessication pretreatment exhibited an undesirable response to particle bombardment. Bombarded cell suspensions turned brown and necrotic even when plated on hygromycin-free medium. It was apparent that those untreated cell suspensions were more sensitive to the physical injury associated with particle bombardment than callus pieces. It was postulated that plasmolyzed cells may be less likely to extrude their

protoplasm following penetration of the cell by particles (Armaleo et al., 1990; Sanford et al., 1992). For this reason, osmotic treatment enhancing stable transformation of *Zea mays* (A188 x B73) cell suspensions was developed by Vain et al. (1993). The initial osmotic treatment consisted of placement of cell suspensions on as osmoticum-containing medium (0.2 M sorbitol and 0.2 M mannitol) 4 h prior to and 16 h after bombardment. If the osmotic enhancement of transient and stable transformation in the previous study (Vain et al. 1993) was truly due to induction of plasmolysis of the cells which reduced cell damage by preventing extrusion of the protoplasm from bombardment cells, then other treatments inducing plasmolysis should have similar effect. Two simple partial desiccation or dehydration treatments were tested for their effect on stable transformation in the present study. The first treatment consist of placing cell suspensions or calli on a piece of dry filter paper and inducing plasmolysis of target cells by partial dehydration for 6-24 hours. This simple treatment was previously proved conductive to plant regeneration of rice calli (Jain et al., 1996; Rance et al., 1994; Tsukahara and Hirosawa, 1992). The second treatment was placing cell suspensions on callus induction CHU medium containing a higher agarose concentration (1.0% w/v) for 2-14 days because that triggered a mild plasmolysis of cell suspensions and resurrection of numerous embryogenic mini-calli, which are ideal target tissues for particle bombardment. As a result, in terms of number of transformed calli per filter bombarded, a 7.6 to 12.7-fold increase of stable transformation over the control was obtained by dehydration of cells on a piece of Whatman filter for 6-to 12 h (Table 4.3). A prolonged (24 h) treatment did not enhance

Table 4.3. Effect of osmotic treatment on stable transformation of rice anther-derived cell suspensions via particle bombardment (LA9502065).

Treatment	Time	No. of filters bombarded	No. of HygR calli	No. of transformed calli per filter
Control	0 h	12	3	0.3
Dehydration on Whatman filter				
	6 h	24	54	2.3
	12 h	12	45	3.8
	24 h	12	21	1.8
Pre-culture on 1.0% Agarose CHU medium				
	2 d	14	20	1.4
	7 d	12	62	5.2
	14 d	12	23	1.9

the beneficial effect; on the contrary, overdue dehydration of cell suspensions on Whatman filter led to an unrecoverable loss of cell viability (data not shown). Similarly, pre-culture of cell suspensions on 1.0% agarose solidified CHU medium for 2 to 14 days enhanced stable transformation by 4.6- to 17.3-fold. Prolonged culture on 1.0% agarose medium for two weeks prior to bombardment resulted in overgrowth of cell clusters and reduced transformation efficiency. These results demonstrated that osmotic treatments dramatically enhanced stable transformation of rice cell suspensions. The optimal osmotic treatment for a high efficiency transformation of rice anther-derived cell suspensions was preculturing them on 1.0% agarose-solidified CHU medium for 7 days or partial dehydration of cell suspensions on one piece of Whatman filter for 12 hours prior to bombardment.

4.3.4 Effect of bombardment distance on stable transformation of cell suspensions

Bombardment distance specifically refers to the distance between the stop screen and target. Three different distances, i.e., 6, 9, and 13 cm preset in the BioRad PDS/He1000 biolistic system, were evaluated for the stable transformation. For two cell suspension lines tested, 7845 and 7631, the highest numbers of transformed calli (3.3 and 5.3 HygR-calli/filter) were obtained by bombardment with the shooting distance of 13 cm, given the other parameters are fixed as described in the section of Materials and Methods, indicating the bombardment distance must be optimized depending on the type of target tissue. In our previous experiment, 9 cm was found optimal for bombardment of scutellum-derived calli, whereas 6 cm was used for successful bombardment-mediated transformation of barley microspore (Jahne et al., 1994) and immature embryos (Wan and Lemaux, 1994).

Table 4.4. Effect of bombardment distance on stable transformation of rice anther-derived cell suspensions.

Genotype	Shooting distance (cm)	No. of filters bombarded†	No. of HygR calli	No. of transformed calli per filter
7845	6	3	2	0.7
	9	3	5	1.7
	13	3	10	3.3
7631	6	3	1	0.3
	9	3	5	1.7
	13	3	16	5.3

† Approximately one gram fresh weight of cell suspensions were plated evenly in the center of each filter.

4.3.5 Effect of cell age on stable transformation and recovery of transgenic plants

The suitability of any type of target tissues including anther-derived calli or cell suspension is limited by multiple factors. One critical factor is cell age, because the regeneration capacity of cells gradually declines during maintenance, and the risk of somaclonal variation increases with the length of time in culture (Jahne et al., 1994). Albinism, phenotypic abnormalities, or reduced fertility have been reported for transgenic maize (Gordon-Kamm et al., 1990), wheat (Vasil et al., 1992), oat (Somers et al., 1992), barley (Wan and Lemaux, 1994), and rice (Jiang et al., unpublished). Our previous studies demonstrated that cell age between 1 and 4 months had negligible influence on competence of scutellum-derived calli for *Agrobacterium*-mediated transformation. Similarly, calli aged 2 to 6 months and cell suspensions aged 4 to 7 months yielded comparable numbers of stable transformants (Table 4.5). However, more (45) albino but

Table 4.5. Effect of cell age on stable transformation and recovery of transgenic plants (LA9502065).

Target type	Cell age (month)	No. of filters bombarded	No. of HygR-calli	No. of transform-ed calli per filter	No. of transgenic plants		
					Albino	Green	Fertile
Calli	2	6	3	0.5	0	3	2
	3	10	4	0.4	0	2	1
	4	12	11	0.9	0	6	3
	5	12	13	1.1	2	5	1
	6	12	8	0.7	4	4	0
Cell susp.	4	8	20	2.5	2	6	2
	5	10	24	2.4	10	2	0
	6	18	35	1.9	14	5	0
	7	15	32	2.1	13	0	0
	Total	103	150	-	45	33	9

fewer (33) green plants were regenerated with the use of aging calli and cell suspensions as target tissues in the present experiment. Concerning fertility of transgenic plants, only 9 of 33 plants transformed from haploid, anther-derived calli or cell suspensions set seeds in the greenhouse. Calli aged 6 months or cell suspensions aged over 5 months produced albinos and sterile plants only. Obviously, primary anther-derived calli or cell suspensions with minimal subculture time were more suitable for production of normal transgenic plants.

4.3.6 Glufosinate resistance and phenotype of R0 transgenic plants

All hygromycin-resistant plantlets were able to establish roots in the presence of 4 mg L^{-1} bialaphos, indicating these Hygromycin-resistant plants were transgenic plants. Plants were further transferred to a greenhouse and sprayed with 2000 mg L^{-1} glufosinate at the 3- to 4-leaf stage. All plants exhibited resistance which was indicated by fully green leaves or by only small necrotic or brown spots in the leaves 7 days after glufosinate application. As shown at Table 4.5, only 9 of 33 transgenic plants of LA9502065 appeared morphologically normal and fertile. The remaining 24 plants exhibited abnormal phenotypes such as sterility and dwarfism. Similarly, only 4 of 13 R0 transgenic plants of Line 7845 set seeds; the remaining plants exhibited dark green leaves, twisted panicles, and sterility. Presumably, the fertile plants were doubled haploids (DH) arising from haploid cells through spontaneous chromosome doubling, whereas sterile transgenic plants remained haploid.

4.3.7 Southern blot hybridization analysis of R0 plants for the *pat* gene

Integration of the introduced *pat* gene into the genome of transgenic rice plants was shown by Southern blot hybridization reaction of undigested genomic DNA from three R0 plants of Line 7845 (data not shown). When the genomic DNA were digested with *EcoR* I, different hybridization banding patterns were detected among transgenic plants, indicating they were independent transformed lines.

4.3.8 Segregation of glufosinate resistance in R1 plants

To investigate the homozygosity of the *pat* gene in fertile R0 transgenic rice plants, a progeny test for glufosinate resistance was conducted. As expected, no segregation of glufosinate resistance was observed in R1 progeny of six primary plants derived from anther calli of LA9502065 (Table 3.6), indicating that these 6 fertile transgenic rice plants were doubled haploids and homozygous for the introduced *pat* gene. However, three primary plants displayed segregation ratios of 15:1 (L2 and L7) or 3:1 (L9) in their progeny, indicating these 3 fertile transgenic plants were heterozygous for the transgene. Heterozygous integration has been reported from the transformation of haploid microspore-derived protoplast of rice by Chair et al. (1996). The reasons for heterozygosity in the present study is likely related to the developmental state or polidy of target cells at the time of bombardment or DNA integration. Spontaneous chromosome doubling could occur in subcultured cells prior to transformation. For example, the existence of diploid (1.3-2.4%) and even aneuploid (2.8-7.7%) cells in a haploid-predominated (90.2-95%) population were reported in a maize pollen-derived cell line (Gu, 1986). The co-existence of haploid (n=12) and diploid (2n=24) cells at almost

Table 4.6. Progeny segregation for glufosinate resistance of 9 primary fertile transgenic plants.

R0 plant†	No. of plants sprayed	No. of plants resistant	No. of plants susceptible	Segregation ratio
L1	35	35	0	1:0
L2	45	42	3	15:1
L3	35	35	0	1:0
L4	47	47	0	1:0
L5	54	54	0	1:0
L6	54	54	0	1:0
L7	23	20	3	15:1
L8	25	24	1	1:0
L9	77	54	23	3:1
7845-1	21	21	0	1:0
7845-2	26	26	0	1:0
7845-3	30	30	0	1:0
7845-4	24	24	0	1:0

† L1~9 transgenic plants were regenerate from anther-derived calli of LA9502065; 7845-1~5 transgenic plants were regenerated from anther-derived cell suspensions.

the same frequency in rice pollen calli were also documented by Chen (1986). Transformation of those chromosome-doubled cells through derived from haploid cells could only yield undesired primary transgenic plants hemizygous to transgene. A progeny test was therefore an essential step to screen out the doubled haploid transgenic rice plant carrying homozygous transgene. In comparison, no segregation was observed in all R1 progeny lines of Line 7845 (Table 4.6), indicating that genotype is likely one of factors influencing cytogenetic stability of subcultured cell lines. The present study for the first time demonstrated that bombardment of haploid anther-derived calli or cell suspensions is a rapid and efficient approach for production of transgenic rice plants.

CHAPTER 5. SUMMARY AND CONCLUSIONS

A rapid and efficient bombardment-based transformation system was developed for production of transgenic glufosinate resistant elite U.S. rice lines and cultivars. Mature seed-derived embryogenic calli induced and proliferated in an optimized CI medium (Table 2.1) were excellent target tissues to generate transgenic rice plants. The transformation efficiency, ranging from 5% for LA9502065 to 100% for Cocodrie, was sufficient for production of a large quantity of transgenic rice plants and selection of elite transgenic lines. Selection of stable transformants using 50 mg L^{-1} hygromycin or 4 mg L^{-1} bialaphos is essential and efficacious for a high transformation efficiency. Without selection no transgenic rice plant was produced, and reduced selection pressure (25 mg L^{-1} hygromycin or 2 mg L^{-1} bialaphos) resulted in low transformation efficiencies and numerous escapes. All R1 and a majority (67%) of R2 progeny derived from Cocodrie and LA9502065 showed Mendelian segregation for glufosinate resistance. Homozygous transgenic rice lines were recovered based on the homogenous resistance of R2 progeny to glufosinate. Field tests of R3 and R4 progeny of 150 transgenic lines conducted recently in the LSU Rice Research Station at Crowley, LA revealed a wide range of phenotypic variation among transgenic lines in terms of maturity, plant height, and other agronomic traits such as seed color. Further field evaluation and selection of diverse glufosinate resistant, transgenic elite lines are on-going for their potential use in rice breeding program.

As an alternative approach to bombardment, a simple but efficient *Agrobacterium*-mediated transformation system for elite U.S. rice lines was also

established using both scutellum- and anther-derived calli as target tissues. On average, a higher transformation frequency (15%) was obtained with anther-derived calli from both LA9502065 (105/716) and Cocodrie (94/606), compared with those of scutellum-derived calli (10-11%). Callus size rather than cell age significantly affected transformation frequency. Actively growing, embryogenic calli of 2-4 mm in size were optimal target tissues for *Agrobacterium* infection. Cell suspensions were found not suitable for *Agrobacterium* transformation. Careful selection of embryogenic calli size and *Agrobacterium* inoculum is of critical importance for a routine, reproducible rice transformation system mediated by *Agrobacterium*. The optimal density of inoculum for the highest transformation frequency (27%) was 1.5 $x10^9$ cells ml^{-1} with the use of AB medium. Southern blot hybridization analysis in conjunction with progeny test of transgenic lines indicated that T-DNA of pTOK233 was stably integrated into rice genome. A segregation ratio of 3:1 for hygromycin resistance was observed in the majority of transgenic lines. Preliminary field studies demonstrated that transgenic rice developed by the *Agrobacterium* approach were relatively uniform in terms of six major agronomic traits, including maturity and fecundity. Plot tests should be conducted in the future to further evaluate field performance of these transgenic rice lines.

In addition, a novel rice transformation system for rapid recovery of transgenic rice plants homozygous for transgenes was established by using haploid anther-derived calli or cell suspensions as target tissues for particle bombardment. One of the prerequisites for production of fertile double haploid transgenic rice plants was using the primary anther-derived calli or cell suspensions with minimal subculture time (≤ 5

months) as target tissues. The CHU medium (Chu et al., 1997) proved suitable for callus induction from anthers of U.S. elite rice lines. Two osmotic treatments prior to bombardment dramatically enhanced stable transformation of rice cell suspensions derived from anther calli (Table 4.3). More sterile than fertile transgenic rice plants were obtained from haploid tissues by both bombardment- and *Agrobacterium*-mediated transformation. Nevertheless, progeny tests revealed that double haploid transgenic rice plants homozygous for the *pat* gene were generated among the primary transgenic rice plants. Our research demonstrated for the first time the competence of anther cultures as target tissues for particle bombardment- and *Agrobacterium*-mediated transformation, though problems of albinism and sterility of regenerated plants should be addressed in future studies to utilize the potential advantages of anther-derived calli or cell suspensions as target tissues in routine transformation work.

REFERENCES

Abdullah, R., E.C. Cooking, and J.A. Thompson. 1986. Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Bio/Technology 4:1087-1090.

Abe T., and Y. Futsuhara. 1986. Genotypic variability for callus formation and plant regeneration in rice (*Oryza sativa* L.). Theor. Appl. Genet. 72:3-10.

Abedinia, M., R.J. Henry, A.B. Blakeney, and L. Lewin. 1997. An efficient transformation system for the Australian rice cultivar, Jarrah. Aust. J. Plant Physiol. 24:133-141.

Abel, P.P., R.S. Nelson, B. De, N. Hoffmann, S.G. Rogers, R.T. Fraley, and R.N. Beachy. 1986. Delay of disease development of in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743.

Aldemita, R.R., and T.K. Hodges.1996. *Agrobacterium tumefaciens*-mediated transformation of *japonica* and *indica* rice varieties. Planta 199:612-617.

Altenbach, S.B., K.W. Pearson, G. Meeker, L.C. Staraci, and S.S.M. Sun.1989. Enhancement of the methionine content of seed proteins by the expression of a chimeric gene encoding a methonine content in transgenic plants. Plant Mol. Biol. 13:513-522.

Arencibia, A., E Gentinetta, E. Cuzzoni, S. Castiglione, A. Kohli, P. Vain, M. Leech, P. Christou, and G. Sala. 1998. Molecular analysis of the genome of transgenic rice (Oryza sativa L.) Plants produced by particle bombardment or intact cell electroporation. Mol. Breed. 4:99-109.

Armaleo, D.G.., N. Ye, T.M. Klein, K.B. Shark, J.C. Sanford, and S.A. Johnston. 1990. Biolistic nuclear transformation of *Saccaromyces cerevisiae* and other fungi. Curr. Genet. 17:97-103.

Ausubel, F.M., R. Bent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and D Struhl. 1992. Short protocols in molecular biology, 2nd editon. John Wiley & Sons, New York.

Ayers, N.M., and W.D. Park. 1994 Genetic transformation of rice. Crit. Rev. Plant Sci. 13:219-239.

Bajaj, Y.P.S. 1991. Biotechnology in Agriculture and Forestry, Vol.12. Rice. Springer-Verlag, Berlin, Heidelberg.

Balconi, C., I. Perugini, S. Castelletti, A. Reali, S. Russo, M.T. Chan, and E. Lupotto. 1998. *Agrobacterium tumefaciens*-mediated trasformation of rice (*Oryza sativa* L. ssp. *japonica*) Italian cultivars. 1. Interaction among *Agrobacterium* strains and rice genotypes in embryogenic callus of somatic and gametic origin. Journal of Genetics and Breeding 52:313-323.

Barton, K. A., A.N. Binns, A.J.M. Matzke, and M.D. Chilton. 1983. Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA and transmission of T-DNA to R1 progeny. Cell 32:1033-1043.

Becker, D., R. Brettschneider, and H. Lorz.1994. Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant J. 5:299-307.

Bidney, D., C. Scelonge, J. Martich, M.Burrus, L.Sims, and G. Huffman.1992. Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. Plant Mol. Biol. 18:301-313.

Botterman, J., and J. Leeman. 1988. Engineering herbicide resistance in plants. Trends Genet. 4:219-222.

Bower, R., and R. Birch. 1992. Transgenic sugarcane plants via microprojectile bombardment. Plant J. 2: 409-416.

Bytebier, B., F. Deboeck, H. DeGrave, M. Van Montagu, and J. P. Hernalsteens. 1987. T-DNA organization in tumor cultures and transgenic plants of the monocotyledon *Asparagus officinalis*. Proc. Natl. Acad. Sci. USA 84:5345-5349.

Cangelosi, G. A., R.G. Ankenbauer, and E.W. Nester.1990. Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proc. Natl. Acad. Sci. USA 87:6708-6712.

Cao, J., X. Duan, D. McElroy, and R. Wu.1992. Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Rep. 11: 586-591.

Caplan, A., L. Herrera-Estrella, D. Inze, E. Van Haute, M. Van Montagu, J. Schell, and P. Zambryski.1983. Introduction of genetic material into plant cells. Science 220: 815-821.

Casas, A.M., A.K. Kononowicz, U.B. Zehr, D.T. Tomes, J.D. Axtell, L.G. Butler, R.A. Bressan, and P.M. Hasegawa. 1993. Transgenic sorghum plants via microprojectile bombardment. Proc. Natl. Acad. Sci. USA. 90:11212-11216.

Chair, H., T. Legavre, and E. Guiderdoni. 1996. Transformation of haploid, microspore-derived cell suspension protoplasts of rice (*Oryza sativa* L.). Plant Cell Rep. 15:766-770.

Chan, M.T., H.H Chang, S.L. Ho, W.F. Tong, and S.M. Yu. 1993. *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α-amylase promoter/β-glucuronidase gene. Plant Mol. Biol. 22:491-506.

Chen, J. L., and W.D. Beversdorf. 1994. A combined use of microprojectile bombardment and DNA imbibition enhances transformation frequency of canola *(Brassica napus* L.). Theor. Appl. Genet. 88: 1887-192.

Chen, L., S. Zhang, R.N. Beachy, and C.M. Fauquet.1998. A protocol for consistent, large-scale production of fertile transgenic rice plants. Plant Cell Rep.18:25-31.

Chen, Y. 1986. The inheritance of rice pollen plant and its application in crop improvement. p118-136. *In* H. Hu and H.Y.Yang (ed.) Haploid of higher plants in vitro. Chinese Academy of Publishers, Beijing, Springer-Verlag, Berlin..

Cheng, M., J.E. Fry, S. Pang, H. Zhou, C.M. Hironaka, D.r. Duncan, T. Coner, and Y.Wan. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens.* Plant Physiol. 115:971-980.

Cheng, X., R. Sardana, H. Kaplan, and I. Altosaar. 1998. *Agrobacterium*-transformed rice plants expressing synthetic *cryIA*(b) and *cryIA*(c) genes are highly toxic to striped stem borer and yellow stem borer. Proc. Natl. Acad. Sci. USA 95:2767-2772.

Chilton, M.D., T.C. Currier, S.K. Farrand, A.J. Bendich, M..P. Gordon, and E.W. Nester.1974. *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc. Natl. Acad. Sci. USA. 71: 3672-3676.

Christensen A.H., R.A. Sharrock, and P. H. Quail. 1992. Maize ubiquitin genes: structure, thermal perturbation of expression and transcript slicing, and promoter activity following transfer to protoplast by electroporation. Plant Mol. Biol. 18:675-689.

Christou, P. 1995. Strategies for variety-independent genetic transformation of important cereals, legumes and woody species utilizing particle bombardment. Euphytica 85:13-27.

Christou, P. 1997. Rice transformation: bombardment. Plant Mol. Biol. 35:197-203.

Christou, P., T.L. Ford, and M. Kofron. 1991. Production of transgenic rice *(Oryza sative* L.) Plants from agronomically important *indica* and *japonica* varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology 9:957-962.

Christou, P., T.L. Ford, and M. Kofron. 1992. The development of a variety-independent gene-transfer method of rice. Trends Biotech. 10:239-246.

Christou, P., D.E. McCabe, B.J. Martinell and W.F. Swain. 1990. Soybean genetic engineering-Commercial production of transgenic plants. Trends Biotech. 8: 145-151.

Chu, C.C., C.C. Wang, C.S. Sun, C. Hsu, K.C. Yin, C.Y. Chu, and F.Y. Bi. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sinica 18:659-668.

Chu, Q.R., H.X. Cao, and S. Linscombe. 1997. A novel medium for induction of embryogenic callus in rice anther culture of Southern U.S. crosses. Rice Biotechnology Quarterly. 32:19-20.

Comai, L., D. Facciotti, W.R. Hiatt, G. Thompson, R.E. Rose, and D.M. Stalker. 1985. Expression in plants of a mutant *aroA* gene form *Salmonella typhimurium* confers tolerance to glyphosate. Nature 317, 741-744.

Comai, L. and Stalker, D. 1986. Mechanism of action of herbicides and their molecular manipulation. Plant Mol. Cell Biol.3:166.

Cooley, J., T. Ford, and P. Christou.1995. Molecular and genetic characterization of elite transgenic rice plants produced by electric-discharge particle acceleration. Theor. Appl. Genet. 90:97-104.

Cornejo, M. J., D. Luth, K.M. Blankenship, O.D. Anderson, and A.E. Blechl.1993. Activity of a maize ubiquitin promoter in transgenic rice. Plant Mol. Biol.23: 567-581.

Cuozzo, M., K.M. O'Connell, W. Kaniewski, R.X. Fang, N.H. Chua, and N.E. Tumer. 1988. Virual protection in transgenic tabacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. Bio/Technology 6: 549-555.

Dale, P. D. and Kinderlerer, J. 1995. Safety in the contained use and the environmental release of transgenic crop plants. p36-63. *In* G.T. Tzotzos (ed.) Genetically modified organisms, a guide to biosafety. CAB International, United Kingdom.

Datta, K., A. Vasquez, J. Tu, L. Torrizo, M.F. Alam, N. Oliva, E. Abrigo, G.S. Khush, and S.K. Datta. 1998. Constitutive and tissue-specific differential expression of the *cryIA*(b) gene in transgenic rice plants conferring resistance to rice insect pest. Theor. Appl. Genet. 97:20-30.

Datta, K., R.Velazhahan, N. Oliva, I. Ona, T. Mew, G.S. Khush, S. Muthukrishnan, and S.K. Datta. 1999. Over-expression of the cloned rice thaumatin-like protein (PR-5) gene

in transgenic rice plants enhances environmental friendly resistance to Rhizoctonia solani causing sheath blight disease. Theor. Appl. Genet. 98:1138-1145.

Datta, S.K. 1998. Transgenic cereals: *Oryza sativa* (rice). *In* I.K. Vasil (ed.) Molecular Improvement of Cereal Crops. Vol. 5. Kluwer Academic Publishers, The Netherlands, in press.

Datta, S.K., K. Datta. N. Soltanifar, G. Donn, and I. Potrykus. 1992. Herbicide-resistant *Indica* rice plants from IRRI breeding line IR72 after PEG-mediated transformation of protoplasts. Plant Mol. Biol. 20:619-629.

Datta, S. K., A. Peterhans, K. Datta, and I. Potrykus. 1990. Genetically engineered fertile Indica-rice recovered from protoplasts. Bio/Technology. 8:736-740.

David, C. C. 1991. The world rice economy: challenges ahead. p1-12. *In* G.S. Khush and G.H. Toenniessen (ed.) Rice Biotechnology: Biotechnology in Agriculture No. 6. CAB International, Wallingford, UK.

De Block, M., J. Botterman, M. Vandewiele, J. Dockx, C. Thoen, V. Gossele, R. Movva, C. Thompson, M. Van Montagu, and J. Leemans. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. 6: 2513-2518.

De Block, M., D. De Brouwer, and P. Tenning. 1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiol. 91: 694-701.

De Clercq, A., M. Vandewiele, J. Van Damme, P. Guerche, M. Van Montagu, J. Vandekerckhove, and E. Krebbers. 1990. Stable accumulation of modified 2S albumin seed storage proteins with higher methionine content in transgenic plants. Plant Physiol. 94:970-979.

De Greef, W., R. Delon, M. De Block, J. Leemans, and J. Botterman. 1989. Evaluation of herbicide resistance in transgenic crops under field conditions. Bio/Technology 61-64.

Dellaporta, S. L., J. Wood, and J. B. Hicks. 1984. Maize DNA miniprep. p.36-37. *In* R. Malmberg et al.(ed.) Molecular Biology of Plants: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

D'Halluin, K., J. Botterman, and W. De Greef. 1990. Engineering of herbicide-resistant alfalfa and evaluation under field conditions. Crop Sci. 30:866-871.

Dong, J., W. Teng, W.G. Buchholz, and T.C. Hall. 1996. *Agrobacterium tumefaciens*-mediated transformation of Javanica rice. Mol. Breed. 2:267-276.

Donn, G., E. Tischer, J.A. Smith, and H.M. Goodman. 1984. Herbicide-resistant alfalfa cells: an example of gene amplification in plants. J. Mol. Appl. Genet., 2:621.

Droge, W., I. Broer, and A.Puhler. 1992. Transgenic plants containing the phosphinothricin-N-acetyltransferase gene metabolize the herbicide phosphinothricin (Glufosinate) differently from untransformed plants. Planta 187;142-151.

Duan X.L., X.G. Xian; Q.Z. Xue, M. Abo-El-Saad, D. Xu, and R. Wu. 1996. Transgenic rice plants harboring an introduced potato proteinase inhibitor II gene are insect resistant. Nature Biotechnology. 14:494-498.

Eckes, P., B. Vijtewaal, and G. Donn. 1989. Synthetic gene confers resistance to the broad spectrum herbicide L-phosphinothricin in plants. J. Cell Biochem Suppl. 13 D: 334

Ellis, D.D., D.E. McCabe, S. McInnis, R. Ramachandran, D.R. Russell, K.M. Wallace, B.J. Martinell, D.R. Roberts, K.F. Raffa, and B.H. McCown. 1993. Stable transformation of *Picea glauca* by particle acceleration-A model system for conifer transformation. Bio/Technology 11: 84-89.

Feinberg, A.P., and B. Vogelstein.1983. A technique for radiolabeling DNA restriction endonuclease fragment to high specific activity. Anal. Biochem.132: 6-13.

Fillatti, J.J., J. Kiser, R. Rose, and L. Comai. 1987. Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Bio/Technology 5: 726-730.

Finer, J. J. and McMullen, M. D. 1990. Transformation of cotton *(Gossypium hirsutum* L.) via particle bombardment. Plant Cell Rep. 8: 586-589.

Fischhoff, D. A., K.S. Bowdish, F.J. Perlak, P.G. Manone, S.M. McCormick, J.G., Niedermeyer, D.A. Fean, K. Kusano-Kretzmer, E.J. Mayer, D.E. Rochester, S.G. Rogers, and R.T. Fraley. 1987. Insect tolerant transgenic tomato plants. Bio/Technology 5:807-813.

Fitch, M.M.M., R.M. Manshardt, D. Gonsalves, J.L. Slightom, and J.C. Sanford. 1990. Stable transformation of papaya via microprojectile bombardment. Plant Cell Rep. 9:189-194.

Fraley, R.T., S.G. Rogers, R.B. Horsch, P.R. Sanders, J.S. Flick, S.P. Adams, M.L. Bittner, L.A. Brand, C.L. Fink, J.S. Fry, G.R.Galluppi, S.B. Goldberg, N.L. Hoffman, and S.C. Woo. 1983. Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA 80: 4803-4807.

Franks, T., and R.G Birch. 1991. Gene transfer into intact sugarcane cells using microprojectile bombardment. Austral. J. Plant Physiol. 18:471-480.

Fromm, M.E., L.P. Taylor and V. Walbot. 1990. Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Bio/Technology 8:833-839.

Fujimoto, H., K. Itoh, M. Yamamoto, J. Kozuka, and K. Shimamoto. 1993. Insect resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis*. Bio/Technology 11: 1151-1155.

Fujimura, T., M. Sakurai, H. Akagi, T. Negishi, and A. Hirose. 1985. Regeneration of rice plants from protoplasts. Plant Tissue Culture Lett. 2:74-75.

Fukuoka, H., T. Ogawa, M. Matsuoka, Y. Ohkawa, and H. Yano. 1998. Direct gene delivery into isolated microspores of rapeseed (*Brassica napus* L.) and the production of fertile transgenic plants. Plant Cell Rep. 17 :323-328.

Gallo-Meagher, M., and J.E. Irvine. 1996. Herbicide resistant transgenic sugarcane plants containing the *bar* gene. Crop Sci. 36:1367-1374.

Gambley, R.L., R. Ford, and G.R. Smith.1993. Microprojectile transformation of sugarcane meristems and regeneration of shoots expressing β-glucuronidase. Plant Cell Rep. 12:343-346.

Ghosh Biswas, G.C., P.K. Burkhardt, J. Wunn, A. Kloti, and I. Potrykus. 1994. Fertile indica rice plants regenerated from protoplasts from scutellar tissue of immature embryos. Plant Cell Rep. 13: 528-532.

Ghosh Biswas, G.C., D.F. Chen, and M.C. Elliott. 1998. A routine system for generation of transgenic rice (*Oryza sativa* L.) plants by microprojectile bombardment of embryogenic cell clusters. Plant Sci. 133:203-210.

Ghosh Biswas, G.C., and F.J. Zapata. 1993. High-frequency plant regeneration from protoplasts of indica rice (*Oryza sativa* L.) using maltose. J. Plant Physiol. 141:470-475.

Gordon-Kamm, W. J., T. M. Spencer, M.L. Mangano, T.R. Adams, R.J. Daines, W.G. Start, J.V.O'Brian, S.A. Chambers, J.W.R. Adams, N.G. Willets, T. B. Rice C.J. Mackey, W. Krueger, A.R. Kausch, and P.G. Lemaux. 1990. Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603-618.

Gould, J., M. Devery, O. Hasegawa, E.C. Ulian, G. Peterson, and R.H. Smith. 1991. Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. Plant Physiol. 95: 426-434.

Graves, A.G., and S.L. Goldman.1987. *Agrobacterium tumefaciens* mediated transformation of the monocot genus *Gladiolus*: detection of the expression of T-DNA-encoded genes. J. Bacteriol.169:1745-1746.

Gressal, J. 1993. Advances in achieving the needs for biotechnologically-derived herbicide resistant crops. p155-198. *In* J. Junick (ed.) Plant Breeding Reviews 11. John Wiley & Son Inc., NewYork.

Griffin, J.L., and S. Micinski. 1998. Transgenic technology: plants with 'designer gene' fend off pests. Louisiana Agriculture 41(4): 18-20.

Grimsley, N. H., C. Ramos, T. Hein, and B. Hohn. 1988. Meristematic tissues of maize plants are most susceptible to Agroinfection with maize streak virus. Bio/Technology 6: 185-189.

Gritz, L., and J. Davies. 1983. Plasmid-encoded hygromycin B resistance : the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene 25: 179-188.

Gu, M.G. 1986. Cytogenetic stability and variability of calli and cell clones originateing from maize pollen and their regenerated plants. p79-90. *In* Hu, H. and H.Y.Yang (ed.) Haploid of higher plants in vitro. Chinese Academy of Publishers, Beijing, Springer-Verlag, Berlin.

Hagio,T., T. Hirabayashi, H. Machii, and H. Tomotsune. 1995. Production of fertile transgenic barley *(Hordeum vulgare* L.) plant using the hygromycin-resistance marker. Plant Cell Rep. 14:329-334.

Harrison, B.D., M.A. Mayo, and D.C. Baulcombe. 1987. Virus resistance in transgenic plants that express cucumber mosaic virus satellite RNA. Nature 328:799-802.

Haughn, G.W., J. Smith, B. Mazur, and C. Somerville. 1988. Transformation with a mutant Arabidopsis acetolactate synthase gene renders tabacco resistant to sulfonylurea herbicides. Mol. Gen. Genet. 211 266-271.

Hayashimoto, A., Z. Li, and N. Murai.1990. A polyethlene glycol-mediated protoplasts transformation system for production of fertile transgenic rice plants. Plant Physiol. 93: 857-983.

Herrera-Estrella, L., A. Depicker, M. Van Montagu, and J. Schell.1983. Expression of chimeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature 303:209-213.

Hess, D., K. Dressler, and R. Nimmrichter. 1990. Transformation experiments by pipetting *Agribacterium* into the spikelets of wheat (*Triticum aestivum* L.). Plant Sci. 72:233-244.

Hidler, V.A., A.M.R. Gatehouse, S.E. Sheerman, R.F. Barker, and D. Boulter. 1987. A novel mechanism of insect resistance engineered into tobacco. Nature 330: 160-163.

Hiei, Y., T. Komari, and T. Kubo.1997. Transformation of rice mediated by *Agrabacterium tumefaciens*. Plant Molecular Biology 35:205-218.

Hiei, Y., S. Ohta, T. Komari, and T. Kumashiro. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. The Plant J. 6: 271-282.

Hoekema, A., P.R. Hirsch, P.J.J. Hooykaas, and R.A. Schilperoort. 1983. A binary plant vector strategy based on separation of *vir* and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303;179-180.

Hoeven, C.V., A. Dietz, and J. Landsmann. 1994. Expression of phosphinothrin acetyltransferase from the root specific par promoter in transgenic tabacco plants is sufficient for herbicide tolerance. Plant Cell Rep.14: 165-170.

Hooykaas, P.J.J., and R.A. Schilperoort. 1992. *Agrobacterium* and plant genetic engineering. Plant Mol. Biol. 19: 15-38.

Iida, A., T. Yamashita, Y. Yamada, and H. Morikawa. 1991. Efficiency of particle bombardment-mediated transformation is influenced by cell cycle stage in synchronized cultured cells of tobacco. Plant Physiol. 97:1585-1587.

Ishida, Y., H. Saito, S. Ohta, Y. Hiei, T. Komari, and T. Kumashiro. 1996. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnology 14: 745-750.

Izawa, T. and K. Shimamoto. 1996. Becoming a model plant: the importance of rice to plant science. Trends Plant Sci. 1:95-99.

Jahne, A., D. Becker, R. Brettschneider, and H. Lorz.1994. Regeneration of transgenic, microspore-derived, fertile barley. Theor. Appl. Genet. 89:525-533.

Jahne, A., D. Becker, and H. Lorz.1995. Genetic engineering of cereal crop plants: a review. Euphytica 85:35-44.

Jain, R.K., S. Jain, B. Wang, and R. Wu. 1996. Optimization of biolistic method for transient expression and production of agronomically useful transgenic Basmati rice plants. Plant Cell Rep. 15: 963-968.

James, D. J., S. Uratsu, J. Cheng, P. Negri, P. Viss, and A.M. Dandekar.1993. Acetosyringone and osmoprotectants like betaine or proline synergistically enhance *Agrobacterium*-mediated transformation of apple. Plant Cell Rep. 12: 559-563.

Jefferson, R.A.. 1987. Assaying chimeric genes in plants: the GUS fusion system. Plant Mol. Biol. Rep.5:387-405.

Jefferson, R.A., T.A. Kavanagh, and M.W. Bevan. 1987. GUS fusion: β-glucuronidase as a selective and versatile gene fusion marker in higher plants. EMBO J. 6: 3901-3907.

Joao, K.H.L. and Brown, T.A. 1993. Enhanced transformation of tomato co-cultivation with *Agrobacterium tumefaciens* C58C1Ri(r)::pGSFR1161 in the presence of acetosyringone. Plant Cell Rep. 12:422-425.

Jones, H., M.J. Tempelaar, and M.G.K. Jones.1987. Recent advances in plant electroporation. Oxford Surveys of Plant Mol.& Cell Biol. 4:347-357.

Khan, M.R.I., L.M. Tabe, L.C. Heath, D. Spencer, and T.J.V. Higgins. 1994. *Agrobacterium*-mediated transformation of subterranean clover *(Trifolium subterraneum* L.). Plant Physiol. 105:81-88.

Khush, G.S. 1997. Origin, dispersal, cultivation and variation of rice. Plant Mol. Biol. 35:25-34.

Khush, G.S., and S. S. Virmani. 1996. Haploids in plant breeding. p11-34. *In* J.S. Mohan et al. (ed.) *In vitro* haploid production in higher plants. vol. 1. Kluwer Academic Publ., Dordrecht, the Netherlands.

Klee, H., R. Horsch, and S. Rogers.1987. *Agrobacterium*-mediated plant transformation and its further application to plant biology. Annu. Rev. Plant Physiol., 38:467.

Klein, T.M., R. Arentzen, P.A. Lewis, and S. Fitzpatrick-McElligot. 1992. Transformation of microbes, plants, and animals by particle bombardment. Bio/Technology 10:286-291.

Klein, T.M., M.. Fromm, A. Weissinger, D. Tomes, S. Schaaf, M. Sletten, and J.C. Sanford.1988. Factors influencing gene delivery into *Zea mays* cells by high velocity microprojectiles. Bio/Technology 6:559-563.

Klein, T.M., L. Kornstein, J.C. Sanrod, and M.E. Fromm. 1989. Genetic transformation of maize cells by particle bombardment. Plant Physiol. 91:440-444.

Klein, T.M., E.D. Wolf, R. Wu, and J.C. Sanford. 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. Nature (London) 327:70-73.

Kloti, A., C. Henrich, S. Bieri, X. He, G. Chen, P.K. Burhardt, J. Wunn, P. Lucca, T. Hohn, I. Potrykus, and J. Futterer. 1999. Upstream and downstream sequence elements determine the specificity of the rice tungro bacilliform virus promoter and influence RNA production after transcription initiation. Plant Mol. Biol. 40:249-266.

Kohli, A., D. Gahakwa, P. Vain, D.A. Laurie, P. Christou. 1999.Transgene expression in rice engineered through particle bombardment: Molecular factors controlling stable expression and transgene silencing. Planta 208:88-97.

Kohli, A., M. Leech, P. Vain, D.A. Laurie, and P. Christou. 1998. Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc. Natl. Acad. Sci. USA. 95:7203-7208.

Komari, T., Y. Hiei, Y. Saito, N. Murai, and T. Kumashiro. 1996. Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J. 10:165-174.

Kosugi, S., Y. Ohashi, K. Nakajima, and Y. Arai. 1990. An improved assay for β-glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci.70: 133-140.

Krens, F.A., L. Molendijik, G.J. Wullems, and R.A. Schilperoort. 1982. In vitro transformation of plant protoplasts with Ti-plasmid DNA. Nature 296:72-74.

Ku, M.S.B., S. Agarie, M. Nomura, H. Fukayama, H. Tsuchida, K. Ono, S. Hirose, S. Toki, M. Miyao, and M. Matsuoka. 1999. High-level expression of maize phosphoenolpyruvate carboxylase in transgenie rice plants. Nature Biotechnology 17: 76-81.

Kumpatla S.P., W. Teng, W.G. Buchholz, and T.C. Hall. 1997. Epigenetic transcriptional silencing and 5-Azacytidine-mediated reactivation of a complex transgene in rice. Plant Physoil. 115:361-373.

LaRossa, R.A., and S.C. Falco. 1984. Amino acid biosynthetic enzymes as targets of herbicide action. Trends in Biotechnology 2:158-161.

Last, D., R.I.S. Brettell, D.A. Chamberlain, A.M. Chaudhury, P.J. Larkin, E.L. Marsh, W.J. Peacock, and E.S. Dennis. 1991. pEmu: an improved promoter for gene expression in cereal cells. Theor. Appl. Genet. 81:581-188.

Lawson, C., W. Kaniewski, L. Haley, R. Rozman, C. Newell, P. Sanders, and N.E. Tumer.1990. Engineering resistance to mixed virus infection in a commercial potato cultivar: residtance to potato virus X and potato virus Y in transgenic Russet Burbank. Bio/Technology 8:127-134.

Lee, K.Y., J. Townsend, J. Tepperman, M. Black, C.F. Chui, B. Mazur, P. Dunsmuir, and J. Bedbrook. 1988. The molecular basis of sulfonylurea herbicide resistance in tobacco. EMBO J. 7: 1241-1248.

Li, L., R. Qu, A. de Kochko, C. Fauquet, and R.N. Beachy. 1993. An improved rice transformation system using the biolistic method. Plant Cell Rep.12:250-255.

Li, Z., M.D. Burow, and N. Murai. 1990. High frequency generation of fertile transgenic rice plants after PEG-mediated protoplast transformation. Plant Mol. Biol. Rep. 8:276-291.

Li, Z., Q. Xie, M..C. Rush, and N. Murai.1992. Fertile transgenic rice plants generated via protoplasts from the U.S. cultivar Labella. Crop Sci 32:810-814.

Lin, W., C.S. Anuratha, K. Datta, I. Potrykus, S. Muthukrishnan and S. K. Datta. 1995. Genetic engineering of rice for resistance to sheath blight. Bio/Technology 3:686-691.

Lindsey, K. 1992. Genetic manipulation of crop plants. J. Biotechnol. 26:1-28.

Linsmaier, E.M., and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant.18:100-127.

Lyon, B.R., D.J. Llewellyn, J.L. Huppatz, E.S. Dennis, and w.J. Peacock. 1989. Expression of a bacterial gene in transgenic tabacco plants confers resistance to 2,4-dichlorophenoxyacetic acid. Plant Mol. Biol. 13: 533-540.

Maqbool, S.B., T. Husnain, S. Riazuddin, L. Masson, and P. Christou.1998. Effective control of yellow stem borer and rice leaf folder in transgenic rice indica varieties Basmati 370 and M7 using the novel delta-endotoxin cry2A *Bacillus thuringiensis* gene. Mol. Breed. 4:501-507.

Mazur, B.J., and S.C. Falco. 1989. The development of herbicide resistance crops. Annual. Rev. Plant Physiol. Plant Mol. Biol. 40:441-470.

McCabe, D. E., and B.J. Martinell. 1993. Tranformation of elite cotton cultivars via particle bombardment of meristems. Bio/Technology 11:596-598.

McCabe, D.E., W.F. Swain, B.J. Martinell, and P. Christou. 1988. Stable transformation of soybean (Glycine max) by particle acceleration.. Bio/Technology 6:923-926.

McElroy, D.,W. Zhang, J. Cao, and R. Wu.1990. Isolation of an efficient actin promoter for use in rice transformation. The Plant Cell2:163-171.

McKenzie, K.S., C.N. Bollich, J.N. Rutger, and K. A. K. Moldenhauer. 1987. Rice. p487-532. *In* W.R. Fehr (ed.) Principle of cultivar development. Vol. 2. Crop species. Macmillan Publishing Company, New York.

Mendel, R.R., B. Muller, J. Schulze, V. Kolesnikov, and A. Zelenin.1989. Delivery of foreign genes to intact barley cells by high velocity microprojectiles. Theor. Appl. Genet. 78:31-34.

Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mooney, P.A., P.B. Goodwin, E.S. Dennis, and D.J. Llewellyn.1991. *Agrobacterium tumefaciens*-gene transfer into wheat tissues. Plant Cell, Tissue and Organ Culture 25:209-218.

Morrish, F., D.D. Songstad, C.L. Armstrong, and M. Fromm. 1993. Microprojectile bombardment: a method for the production of transgenic cereal crop plants and the functional analysis of genes. p.133-171. *In* A. Hiatt(ed.) Transgenic Plants: fundamentals and applications. Marcel Dekker, Inc., New York.

Murakami, T., H. Anzai, S. Imai, A. Satoh, K. nagaoka, and C.J. Thompson.1986. The bialaphos biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning and characterization of the gene cluster. Mol. Gen. Genet. 205: 42-47.

Murashige,T., and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15:473-479.

Nagy, F., G. Morelli, R.T. Fraley, S.G. Rogers, and N.H. Chua.1985. Photoregulated expression of a pea rbcS gene in leaves of transgenic plants. EMBO J. 2:3063-3068.

Nayak, P., D. Basu, S. Das, A. Basu, D. Ghosh, N.A. Ramakrishnan, M. Ghosh, and S.K. Sen.1997. Transgenic elite indica rice plants expressing *CrylAc* delta-endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). Proc. Natl. Acad. Sci. USA. 94: 2111- 2116.

Nehra, N.S., R.N. Chibbar, N. Leung, K. Caswell, C. Mallard, L. Steinhauer, M. Baga, and K.K. Kartha.1994. Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. Plant J. 5:285-297.

Nelson, R.S., S.M. McCormick, X. Delannay, P. Dube, J. Layton, E.J. Anderson, M. Kaniewska, R.K. Proksch, R.B. Horsch, S.G. Rogers, R.T. Fraley, and R.N. Beachy. 1988. Virus tolerance, plant growth, and field performance of transgenic tomato plants expressing coat protein from tobacco mosaic virus. Biotechnology 6:403-409.

Nishihara, M., M. Seki, M. Kyo, K. Irifune, and H. Morikawa. 1995. Transgenic haploid plants of Nicotiana rustica produced by bombardment-mediated transformation of pollen. Transgenic Res. 4:341-348.

Niizeki, H., and K. Oono. 1968. Induction of haploid rice plant from anther culture. Proc. Jpn Acad. 44:554-557.

Oard, J.H., S.D. Linscombe, M.P. Braveman, F. Jordari, D.C. Blouin, M. Leech, A. Kohli, P. Vain, J.C. Cooley, and P. Christou. 1996. Development, field evaluation, and agronomic performance of transgenic herbicide resistance rice. Mol. Breed. 2:259-368.

Odell, J.T., F. Nagy, and N.H. Chua. 1985. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810-812.

Ohira, K., K. Ojima, and A. Fijiwara. 1973. Studies on the nutrition of rice cell culture I. A simple, defined medium for rapid growth in suspension culture. Plant Cell Physiol. 14:1113-1121.

Ohta, S., S. Mita, T. Hattori, and K. Nakamura. 1990. Construction and expression in tobacco of a β-glucurodinase (GUS) reporter gene containing an intron within the coding sequence. Plant Cell Physiol. 31:805-813.

Otten, L., H. De Greve, J.P. Hernalsteens, M.. Van Montagu, O. Schieder, J. Straub, and J. Schell. 1981. Mendelian transmission of genes introduced into plants by the Ti plasmids of *Agrobacterium tumefaciens*. Mol. Gen. Genet. 183: 209-213.

Ou-Lee, T.M., R. Turgeon, and R. Wu. 1986. Expression of a foreign gene linked to either a plant virus or a Drosophila promoter after electroporation of protoplasts of rice, wheat, and sorghum. Proc. Natl. Acad. Sci. USA 83:6815-6819.

Owens, L.D. 1995. Overview of gene availability, identification, and regulation. HortSci. 30:957-961.

Park S.H., S.R.M. Pinson, and R.H. Smith.1996. T-DNA integration into genomic DNA of rice following *Agrobacterium* inoculation of isolated shoot apices. Plant Mol. Biol. 32: 1135-1148.

Peng, J., L.A. Lyznik, L. Lee, and T.K. Hodges. 1990. Cotransformation of *indica* rice protoplasts with GUS and Neo genes. Plant Cell Rep. 9: 168-172.

Perlak, F.J., R.W. Deaton, T.A. Armstrong, R.L. Fuchs, S.R. Sims, J.T. Greenplate, and D.A. Fischhoff. 1990. Insect resistant cotton plants. Bio/Technology 8: 939-943.

Pinto, Y.M., R.A. Kok, and D.C. Baulcombe.1999. Resistance to rice yellow mottle virus (RYMV) in cultivated African rice varieties containing RYMV transgenes. Nature Biotechnology 17:702-707.

Potrykus, I. 1990. Gene transfer to cereals: an assessment. Bio/Technology 8:535-542.

Potrykus, I., C.T. Harms, and H. Lorz. 1979. Callus formation from cell culture protoplasts of corn (*Zea mays* L.). Theor. Appl. Genet.54:209-214.

Raineri, D.M., P. Buttino, M..P. Gordon, and E.W. Nester. 1990. *Agrobacterium*-mediated transformation of rice (*Oryza sativa*). Bio/Technology 8:33-38.

Rance, I.M., W. Tian, H. Mathews, A. de Kochko, and R.N. Beachy. 1994. Partial desiccation of mature embryo-derived calli, a simple treatment that dramatically enhances the regeneration ability of indica rice. Plant Cell Rep. 13:647-651.

Rashid, H., S. Yokoi, K. Toriyama, and K. Hinata. 1996. Transgenic plant production mediated by *Agrobacterium* in *Indica* rice. Plant Cell Reports 15:727-730.

Rathore, K.S., V.K. Chowdhury, and T.K. Hodges. 1993. Use of *bar* as a selectable marker gene and for the production of herbicide-resistant rice plants from protoplasts. Plant Mol. Biol. 21:871-884.

Rhodes, C.A., K.S. Lowe, and K.L. Ruby.1988a. Plant regeneration from protoplasts isolated from embryogenic maize cell cultures. Bio/Technology 6:56-60.

Rhodes, C.A., D.A. Pierce, J.J. Mettler, D. Mascarenhas, and J.J. Detmer. 1988b. Genetically transformed maize plants from protoplasts. Science 24:204-207.

Ritala, A., K. Aspegren, U. Kurten, M. Salmenkallio-Marttila, L. Mannonen, R. Hannus, V. Kauppinen, T.H. Teeri, and T.M. Enari.1994. Fertile transgenic barley by particle bombardment of immature embryos. Plant Mol. Biol. 24:317-325.

Russell, D. R., K. Wallace, J. Bathe, B.J. Martinell, and D.E. McCabe. 1993. Stable transformation of *Phaseolus vulgaris* via electric discharge-mediated particle acceleration. Plant Cell Rep. 12:165-169.

Russell, J.A., M.K. Roy, and J.C. Sanford. 1992a. Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. Plant Physiol.98: 1050-1056.

Russell, J.A., M.K. Roy, and J.C. Sanford. 1992b . Major improvement in biolistic transformation of suspension-cultured tobacco cells. *In Vitro* Cell. Dev. Biol.28:97-105.

Sambrook, J., E.F. Fritsch, and T. Maniatis . 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sanford, J.C. 1988. The biolistic process. Trends Biotechnol.6:299-302.

Sautter, C., H. Waldner, G. Neuhaus-Url, A. Galli, G. Neuhaus, and I. Potrykus. 1991. Micro-targeting: high efficiency gene transfer using a novel approach for the acceleration of microprojectiles. Bio/Technology 9:1080-1085.

Schlappi, M., and B. Hohn. 1992. Competence of immature maize embryos for *Agrobacterium*-mediated gene transfer. The Plant Cell 4:7-16.

Seki, M., N. Shigemoto, Y. Komeda, J. Imamura, Y. Yamada, and H. Morikawa. 1991. Transgenic Arabidopsis thaliana plants obtained by particle bombardment-mediated transformation. Appl. Microb. and Biotech. 36:228-230.

Serres, R., E. Stang, D. McCabe, D. Russell, D. Mahr, and B. McCown.1992. Gene transfer using electric discharge particle bombardment and recovery of transformed cranberry plants. J. Amer. Soc. Hort. Sci. 117: 174-180.

Shah, D.M., D.M. Hironaka, R.C. Wiegand, E.I. Hardding, G.G. Krivi, and D.C. Tiemaier. 1986a. Structural analysis of a maize gene coding for glutathione-S-transferase involved in herbicide detoxification. Plant Mol. Biol.6:203.

Shah, D.M., R.B. Horsch, H.J. Klee, G.M. Kishore, J.A. Winter, N.E. Tumer, C.M. Hironaka, P.R. Sanders, C.S. Gasser, S. Aykent, N.R. Siegel, S.G. Rogers, and R.T. Fraley. 1986b. Engineering herbicide tolerance in transgenic plants. Science 233:478-481.

Sheikholeslam, S.N., and D.P. Weeks. 1987. Acetosyringone promotes high efficiency transformation of Arabidopsis thaliana explants by *Agrobacterium tumefaciens*. Plant Mol. Biol. 8:291-298.

Shillito, R. D., G.K. Carswell, C.M. Johnson, J.J. DiMaio, and C.T. Harms. 1989. Regeneration of fertile plants from protoplasts of elite inbred maize. Bio/Technology 7:581-587.

Shimamoto, K. 1995. The molecular biology of rice. Science 270:1772-1773.

Shimamoto, K., R. Terada, T. Izawa, and H. Fujimoto.1989. Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338: 274-277.

Shimoda, N., A. Toyoda-Yamamoto, J. Nagamine, S. Usami, M. Katayama, Y. Sakagami, and Y. Machida. 1990. Control of expression of *Agrobacterium* vir genes by synergistic actions of phenolic signal molecules and monosaccharides. Proc. Natl. Acad. Sci. USA 87:6684-6688.

Siriwardana, S. and Nabors, M.W. 1983. Tryptophan enhancement of somatic embryogenesis in rice. Plant Physiol. 73: 143-146.

Sivamani, E., H. Huet, P. Shen, A.O. Ching, A. de Kochko, C. Fauquet, and R. N. Beachy. 1999. Rice plant (*Oryza sativa* L.) containing rice tungro spherical virus (RTSV) coat protein transgenes are resistant to virus infection. Mol. Breed.5:177-185.

Sivamani, E., P. Shen, N. Opalka, R.N. Beachy, C.M. Fauquet. 1996. Selection of large quantities of embryogenic calli form indica rice seeds for production of fertile transgenic plants using the biolistic method. Plant Cell Rep 15:322-327.

Somers, D.A., H.W. Rines, W. Gu, H.F. Kaeppler, and W.R. Bushnell.1992. Fertile, transgenic oat plants. Bio/Technology 10:1589-1594.

Song, W.Y., G.L. Wang, L.L. Chen, H.S. Kim, L.Y. Pi, T. Holsten, J. Gardner, B. Wang, W.X. Zhai, L.H. Zhu, C. Fauquet, and P. Ronald. 1995. Receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science 270:1804-1806.

Songstad , D. D., C.L. Armstrong, W.L. Petersen, B. Hairston, and M.A.W. Hinchee. 1995. Advances in alternative DNA delivery techniques. Plant Cell, Tissue, and Organ Culture 40:1-15.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Spencer, T.M., W.J. Gordon-Kamm, R.J. Daines, W.G. Start, and P.G. Lemaux. 1990. Bialaphos selection of stable transformation from maize cell culture. Theor. Appl. Genet. 79:625-631.

Stachel, S. E., E.Messens, M. Van Montagu, and P. Zambryski.1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium* tumefaciens. Nature 318:624-629.

Stalker, D.M., K.E. McBride, and L.D. Malyj. 1988. Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. Science 242: 419-423.

Stiff, C.M., A. Kilian, H. Zhou, D.A. Kudrna, and A. Kleinhofs.1995. Stable transformation of barley callus using biolistic particle bombardment and the phosphinothricin acetyltransferase (*bar*) gene. Plant Cell, Tissue, and Organ culture 40:243-248.

Stoger, E., C. Fink, M. Pfosser, and E. Hoberle-Bors. 1995. Plant transformation by particle bombardment of embryogenic pollen. Plant Cell Rep. 14:273-278.

Strauch, E., W. Wohlleben, and A. Puhler.1988. Cloning of a phosphinothricin-N-acetyltransferase gene from *Streptomyces viridochromogenes* Tu 494 and its expression in *Streptomyces lividans* and *Escherichia coli*. Gene 63: 65-74.

Streber, W.R., and L. Willmitzer. 1989. Transgenic tobacco plants expressing a bacterial detoxifying enzyme are resistant to 2,4-D. Bio/Technology 7:811-816.

Swaminathan, M.S. 1982. Biotechnology research and third world agriculture. Science 218:967-972.

Tachibana K., T. Watanabe, Y. Sekizawa, and T. Takematsu. 1986. Action mechanism of bialaphos. II. Accumulation of ammonia in plants treated with bialaphos. J. Pest Sci.11: 33-37.

Tang, K., P. Tinjuangjun, Y. Xu, X. Sun, J.A. Gatehouse, P.C. Ronald, H. Qi, X. Lu, P. Christou, and A. Kohli. 1999. Particle bombardment-mediated co-transformation of elite Chinese rice cultivars with genes conferring resistance to bacterial blight and sap-sucking insect pests. Planta 208:552-563.

Taylor, M.G., and I.K. Vasil. 1991. Effect of physical factors, and the histology of transient GUS expression in pearl millet (*Pennisetum glacum* L.) embryos following microprojectile bombardment. Plant Cell Rep. 10:120-125.

Terada, R., and K. Shimamoto. 1990. Expression of CaMV35S-GUS gene in transgenic rice plants. Mol. Gen. Genet. 220:389-392.

Thomas, M.S., and R. B. Flavell. 1990. Identification of an enhancer element for the endosperm-specific expression of high molecular weight glutenin. The Plant Cell. 2:1171-1180.

Thompson, C.J., N. Rao Movva, R. Tizard, R. Crameri, J.E. Davies, M. Lauwereys, and J. Botterman. 1987. Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J. 6: 2519-2523.

Tingay, S., D. McElroy, R. Kalla, S. Fieg, M. Wang, S. Thornton, and R. Brettell. 1997. *Agrobacterium tumefaciens*-mediated barley transformation. Plant J. 11:1369-1376.

Toenniessen, G.H. 1995. The Rockefeller Foundation's international program on rice biotechnology. p.193-212. *In* D.W. Altman and K.N Watanabe (ed.) Plant Biotechnology to Developing Countries. R.G. Landes Company.

Toenniessen, G.H. 1996. Progress in rice biotechnology. p1-11. *In* Proceedings of the fifth annual meeting national rice biotechnology network. IARI, New Delhi.

Toki, S. 1997. Rapid and efficient *Agrobacterium*-mediated transformation in rice. Plant Mol. Biol. Rep.15:16-21.

Toki, S., S. Takamatsu, C. Nojiri, S. Ooba, H. Anzai, M. Iwata, A.H. Christensen, P.H. Quail, and H. Uchimiya. 1992. Expression of a maize ubiquitin gene promoter-*bar* chimeric gene in transgenic rice plants. Plant Physiol.100:1503-1507.

Tomes, D.T., A.K. Weissinger, M. Ross, R. Higgins, B.J. Drummond, S. Schaaf, J. Malone-Schoneberg, M. Staebell, P. Flynn, and J. Anderson.1990. Transgenic tobacco plants and their progeny derived from microprojectile bombardment of tobacco leaves. Plant Mol. Biol. 14:261-268.

Toriyama, K., Y. Arimoto, H. Uchimiya, and K. Hinata.1988. Transgenic rice plants after direct gene transfer into protoplasts. Bio/Technology 6:1072-1074.

Toriyama, K., and K. Hinata. 1985. Cell suspension and protoplast culture in rice. Plant Sci. 41:179-183.

Torrizo, L.B., and F.J. Zapata. 1992. High efficiency plant regeneration from protoplasts of the indica cultivar IR58. J. Expermental Bot. 43:1633-1637.

Tsukahara, M., and T. Hirosawa. 1992. Simple dehydration treatment promotes plantlet regeneration of rice (*Oryza sativa* L.) callus. Plant Cell Rep. 11:550-553.

Tu, J., I. Ona, Q. Zhang, T.W. Mew, G.S. Khush, and S.K. Datta. 1998. Transgenic rice variety 'IR72' with Xa21 is resistant to bacterial blight. Theor. Appl. Genet. 97:31-36.

Tyagi, A.K., A. Mohanty, S. Bajaj, A. Chaudhury, and S.C. Maheshwari. 1999. Transgenic rice: A valuable monocot system for crop improvement and gene research. Critical Reviews in Biotechnology 19:41-79.

Uchimiya, H., T. Fushimi, H. Harada, K. Syono, and Y. Sugawara. 1986. Expression of a foreign gene in callus derived from DNA-treated protoplasts of rice (*Oryza sativa* L.). Mol. Gen. Genet. 16:204-207.

Vacek, M., A. Reynaerts, H. Hofte, S. Jansens, M. De Beuckeleer, C. Dean, M. Zabeau, M. Van Montagu, and J. Leemans. 1987. Transgenic plants protected from insect attack. Nature 328: 33-37.

Vain, P., M.D. McMullen, and J.J. Finer. 1993. Osmatic treatment enhances particle bombardment-mediated transient expression and stable transformation of maize. Plant Cell Rep. 12:84-88.

Vain, P., B. Worland, M.C. Clarke, G. Richard, M. beavis, H. Liu, A. Kohli, M. Leech. J. Snape, P. Christou, and H. Atkinson. 1998. Expression of an engineered cysteine proteinase inhibitor (Oryzacystatin-IΔD86) for nematode resistance in transgenic rice plants. Theor. Appl. Genet. 96:266-271.

Valdez, M., J.L. Cabrera-Ponce, D. Sudhakar, and L. Herrera-Estrella. 1998. Transgenic Central American, West Africa and Asian elite rice varieties resulting form particle bombardment of foreign DNA into mature seed-derived explants utilizing three different bombardment devices. Annals of Botany 82:795-801.

Vasil, I.K. 1987. Developing cell and tissue culture systems for the improvement of cereal and crop plants. J. Plant Physiol. 128:193-218.

Vasil, V., S.M. Brown, D. Re, M.F. Fromm, and I.K. Vasil. 1991. Stably transformed callus lines from microprojectile bombardment of cell suspension cultures of wheat. Bio/Technology 9:743-747.

Vasil, V., A.M. Castillo, M.E. Fromm, and I. Vasil. 1992. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technology 10: 667-674.

Vasil, V., V. Srivastava, A.M. Castillo. M.E. Fromm, I.K. Vasil. 1993. Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. Bio/Technology 11: 1553-1558.

Vernade, D., A. Herrera-Estrella, K. Wang, and M. van. Montagu. 1988. Glycine betaine allows enhanced induction of the *Agrobacterium tumefaciens vir* genes by acetosyringone at low pH. J. Bacteriol. 170:5822-5829.

Vijayachandra, K., K. Palanichelvam, and K. Veluthambi. 1995. Rice scutellum induces *Agrobacterium tumefaciens vir* genes and T-strand generation. Plant Mol. Biol. 29:125-133.

Wan, Y. and P.G. Lemaux. 1994. Generation of large numbers of independently-transformed fertile barley plants. Plant Physiol. 104: 37-48.

Wan, Y., J.M. Widholm, and P.G. Lemaux. 1995. Type I callus as a bombardment target for generating fertile transgenic maize (Zea mays L.). Planta. 196:7-14.

Wang, M.B., N.M. Upadhyaya, R.I.S. Brettell, and P.M.Waterhouse. 1997. Intron-mediated improvement of a selectable marker gene for plant transformation using *Agrobacterium tumefaciens*. Journal of Genetics and Breeding 51:325-334.

Weeks, T. J., O.D. Anderson, and A.E. Blechl. 1993. Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). Plant Physiol. 102: 1077-1084.

Wilde, H.D., R.B. Meagher, and S.A. Merkle. 1992. Expression of foreign genes in transgenic yellow-poplar plants. Plant Physiol. 98:114-120.

Williams, C. E., and P. C. Ronald. 1994 . PCR template-DNA isolated quickly form monocot and dicot leaves without tissue homogenization. Nucleic Acids Res. 22: 1917-1918.

Wohlleben, W., W.Arnold, I. Broer, D. Hillemann, E. Strauch, and A. Puhler. 1988. Nucleotide sequence of the phosphinothricin-N-acetyltransferase gene from *Streptomyces viridochromogenes* Tu 494 and its expression in *Nicotiana tobacum*. Gene 70:25-37.

Wu, C., Y. Fan, C. Zhang, N. Oliva, and S.K. Datta. 1997. Transgenic fertile japonica rice plants expressing a modified *cryIA*(b) gene resistant to yellow stem borer. Plant Cell Rep. 17:129-132.

Wu, H., A.C. McCormac, M.C. Elliott, and D.F. Chen. 1998. *Agrobacterium*-mediated stable transformation of cell suspension cultures of barley (*Hordeum vulgare*):Plant Cell, Tissue and Organ Culture 54:161-171.

Wunn. J., A. Kloti, P.K. Burkhardt, G.C. Ghosh Biswas, K.Launis, V.A. Iglesias, and I. Potrykus. 1996. Transgenic indica rice breeding lines IR58 expressing a synthetic

cryIA(b) gene from *Bacillus thuringiensis* provides effective insect pest control. Bio/Technology 14:171-176.

Xu, D., X. Duan, B. Wang, H. Bimei, T.D. Ho, and R. Wu. 1996. Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficient and salt stress in transgenic rice. Plant Physiol. 110:249-257.

Yamashita T., A. Iida, and H. Moikawa. 1991. Evidence that more than 90% of β-glucuronidase-expressing cells after particle bombardment directly receive the foreign gene in their nucleus. Plant Physiol.97: 829-831.

Yao, Q. A., and K.J. Kasha. 1997. Potential of biolistic transformation of barley microspores based on viability and transient beta-glucuronidase activity. Genome 40:639-643.

Yao, Q.A., E. Simion, M. William, J. Krochko, and K.J. Kasha.1997. Biolistic transformation of haploid isolated microspores of barley (*Hordeum vulgare* L.). Genome 40:570-581.

Ye, G. N., H. Daniell, and J.C. Sanford. 1990. Optimization of delivery of foreign DNA into higher-plant chloroplasts. Plant Mol. Biol.15:809-819.

Yokoi, S., S.I. Higashi, S. Kishitani, N. Murata, and K. Toriyama. 1998. Introduction of the cDNA for Arabidopsis glycerol-3-phosphate acyltransferase (GPAT) confers unsaturation of fatty acids and chilling tolerance of photosynthesis on rice. Mol. Breed. 4:269-275.

Zambryski, P., H. Joose, C. Genetello, J. Leemans, M. Van Montagu, and J. Schell. 1983. *Ti* plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J. 2: 2143-2150.

Zhang, H. M., H. Yang, E.L. Rech, T.J. Golds, A.S. Davis, B.J. Mulligan, E.C. Cocking, and M.R. Davey. 1988. Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. Plant Cell Rep. 7:379-384.

Zhang, S., L. Chen, R. Qu, P. Marmey, R. Beachy, and C. Fauquet.1996. Regeneration of fertile transgenic *indica* (grope 1) rice plants following microprojectile transformation of embryogenic suspension culture cells. Plant Cell Rep.15:465-469.

Zhang, W., and R. Wu. 1988. Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants. Theor. Appl. Genet. 76:835-840.

Zheng, H., Y. Li, Z. Yu, W. Li, M. Chen, X. Ming, R. Casper, and Z. Chen. 1997. Recovery of transgenic rice plants expressing the rice dwarf virus outer coat protein gene (S8). Theor Appl. Genet. 94:522-527.

Zheng, Z., A. Hayashimoto, Z. Li, and N. Murai. 1991. Hygromycin resistance gene cassettes for vector construction and selection of transformed rice protoplasts. Plant Physiol. 97: 832-835.

Zimmerman, U. 1986. Electrical breakdown, electropermerabilization and electrofusion. Rev. Physiol. Biochem. Pharmacol. 105:175-256.

VITA

Junda Jiang was born on October 4, 1964, in a village located in the eastern costal area of Fenghua, Zhejiang Province, China. He was brought up in a rice-growing community by a couple of hard-working and plain parents. In his childhood, he loved stepping in paddy fields of the community, helping his parents transplanting and harvesting rice or in a private plot growing vegetables. He was not a genius but a diligent pupil. He adored teachers and cherished knowledge in a rural elementary and junior high school. At the age of 15, he attended a top senior high school in Fenghua and started learning science in courses of mathematics, physics, chemistry, and biology, as well as English. He found his interests and aptitudes in agriculture and biology. At the age of 19, he enrolled in Zhejiang Agricultural University (ZAU), one of the oldest and top agricultural colleges in the nation. He selected a major for crop breeding and genetics. He was a receipt of the ZAU Scholarship for four consecutive academic years and earned his bachelor of science degree in agronomy in 1987. He continued his study for a master of science degree in crop breeding and genetics in the same college, and minored in plant tissue culture under his major professor and rice breeder Dr. Qingzhong Xue. He earned his master of science degree in 1990. His thesis was "Isolation, culture, and callus regeneration of sunflower protoplasts". Upon graduation, he was employed as a Research Assistant (1990-1992) and Research Associate (1992-1995) by the Agricultural Biotechnology Center, the Shanghai Academy of Agricultural Sciences (SAAS). He participated in several state projects in rice molecular biology and biotechnology including rice anther culture, protoplast culture, genetic transformation, and RFLP

mapping, directed by Dr. Qiren Chu, a well-known rice biotechnologist. During his tenure in SAAS, he published or co-authored seven papers and won one academic paper award by the Chinese Society of Agronomy in 1992. He also participated in a project of commercial micro-propagation of ornamental and medicinal plants. In 1995, he enrolled in the Graduate School of Louisiana State University A&M College, Baton Rouge, and embarked upon a doctoral degree program in rice breeding and genetics under the direction of Dr. Steve D. Linscombe in the Rice Research Station at Crowley and Dr. James H. Oard in the Department of Agronomy on the main campus. In 1998, he presented two papers on rice transformation in the 27th Rice Technical Working Group (RTWG) meeting held in Reno, Nevada. He was honorably nominated by the Department of Agronomy and awarded 1998 G. O. Mott Meritorious Graduate Student Award by Crop Science Society of America in recognition of his outstanding achievements and contributions in developing efficient DNA transformation methods for elite U.S. rice lines while working towards a graduate degree in crop science. He is a member of the American Society of Agronomy, Crop Science Society of America, Gamma Sigma Delta Honor Society of Agriculture and an associate member of Sigma Xi-The Scientific Research Society. He has been employed by the Department of Agronomy as a Research Associate Specialist for rice transformation under the supervision of Dr. James Oard since March 1998. He is currently a candidate for the degree of Doctor of Philosophy in agronomy, which will be conferred in December 1999. He was married to Ying Chen in 1993 and has been given a very blessed daughter Jessie Grace Jiang in May 1998.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Junda Jiang

Major Field: Agronomy

Title of Dissertation: Development of Efficient Rice DNA Transformation Methods and Rapid Field Evaluation of Transgenic Lines

Approved:

(co-chair) _____
Major Professor and Chairman (co-chair)

_____
Dean of the Graduate School

EXAMINING COMMITTEE:

_____

_____

_____

_____

Jos Wooding (dissenting)

_____

_____

_____

_____

Date of Examination:

October 18, 1999